# The role of group I metabotropic glutamate receptors in absence epilepsy

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# The role of group I metabotropic glutamate receptors in absence epilepsy

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## Doctoral Thesis

to obtain the degree of doctor
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according to the decision of the Council of Deans
to be defended in public on Wednesday 8 June 2016
at 12.30 hours

by

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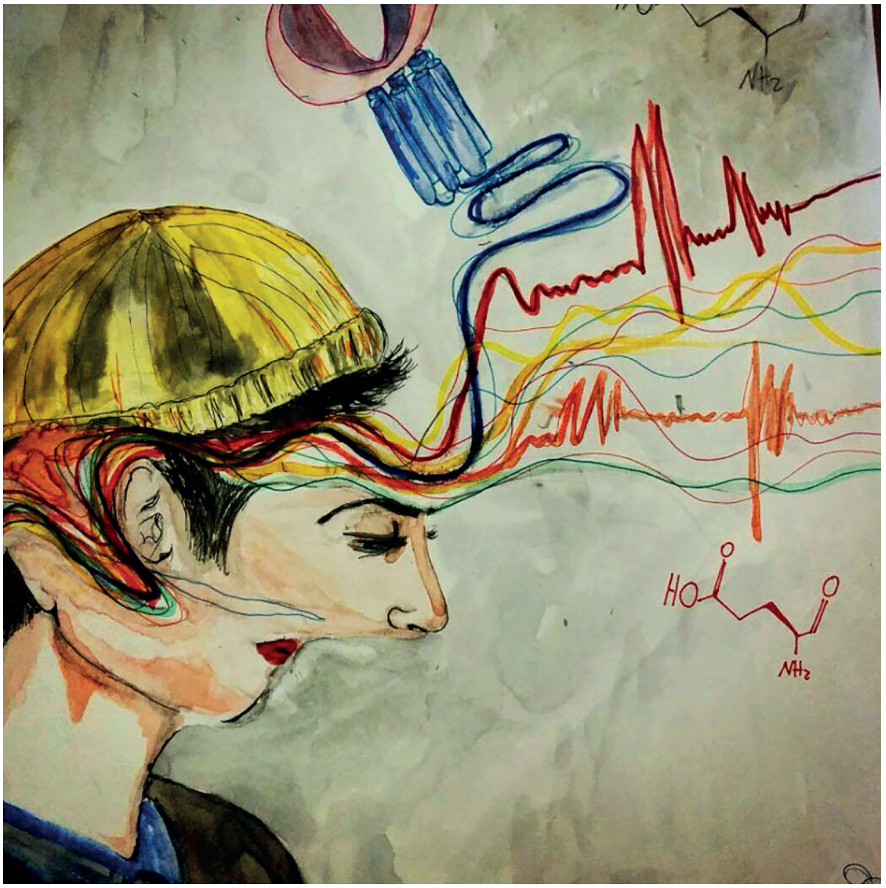



# 1 Introduction

# General Introduction

## Epilepsy

Epilepsy is a chronic disease of the brain characterized by an enduring predisposition to generate epileptic seizures (Fisher et al., 2014). This disease largely influences the patient's life. It is one of the most common neurological disorders requiring long-term health care contact (Forsgren, 1992; Sander et al., 1996). Worldwide approximately 50 million people have epilepsy (Banerjee, 2009). One in 26 people will develop epilepsy during their lifetime (Hesdorffer et al., 2011).

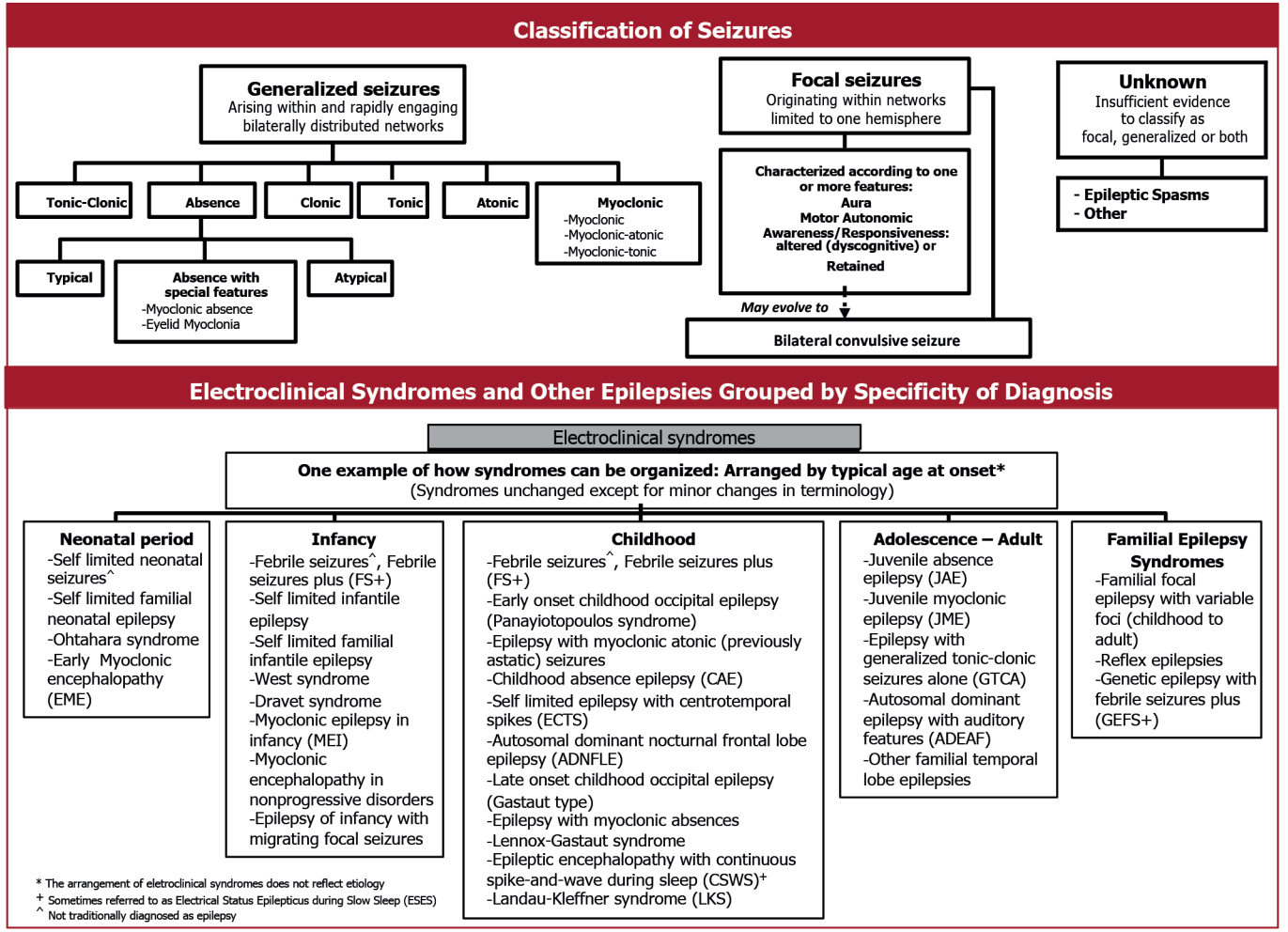
Epilepsy is an expression of various brain disorders – not only a single disease. Consequently, any investigations or treatment needs to be individualized. The epilepsies represent a heterogeneous group of disorders with different electrographic, etiologies, behavioural and seizure patterns. In 1985, the first concept of epileptic syndromes was brought into consideration. Etiology was only ever mentioned briefly in the 1969, 1981, and 1989 International League Against Epilepsy (ILAE) classifications of seizures and syndromes, as well as later publications (Gastaut, 1969; Luders et al., 2004). In the 1989 classification of epilepsies, these classes were given: localization-related epilepsies and syndromes, generalized epilepsies and syndromes, epilepsies and syndromes, undetermined whether focal or generalized, and special syndromes. In its 2010 report the last ILAE Commission confined its revisions to "new terminology and concepts" instead of "proposing a new classification (in the sense of organization) of epilepsies."

This outmost current ILAE definition states that: "Epilepsy is a disorder of the brain characterized by an enduring predisposition to arise epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. In this current classification from 2010 the terms "symptomatic", "idiopathic" and "cryptogenic" have been changed by "structural-metabolic", "genetic" and "unknown" but they do not list etiologic categories any further (see Table 1 middle panel) (Berg et al., 2010).

Epilepsy should primary be classed according to etiology, succeed by a narration of the semiology of the seizure. However, this current classification has also been criticized and authors have deliberated the necessity for a more up-to-date way to the grouping of epileptic seizures and epilepsies (Luders et al., 2012).

In clinical practice the diagnosis of epilepsy is founded on the clinical history and physical inspection. However, it is important to get a detail picture of the seizure's semiology as possible. Ictal symptoms, i.e. symptoms during the seizures, particularly at onset, are determined by the localization of seizure foci. Moreover, it is also significant to evaluate differential diagnoses like syncope, arrhythmia and non-epileptic seizures, since a wrong diagnosis of epilepsy is often common (Chadwick et al., 2002). Interictal electro-encephalogram (EEG) can provide information about the diagnosis of epilepsy, although a normal EEG cannot exclude the diagnosis of epilepsy.

**Table 1** ILAE Proposal for revised Terminology for Organization of Seizures and Epilepsies.



## Major changes in terminology and concepts

| New Term and Concept | Examples | Old Term and Concept |
|---|---|---|
| Etiology (an individual may fit into more than one group) | | |
| **Genetic:** *genetic defect directly contributes to the epilepsy and seizures are the core symptom of the disorder* | *Channelopathies, GLUT1 deficiency, etc* | **Idiopathic:** *presumed genetic* |
| **Structural:** *caused by a structural disorder of the brain* | Tuberous sclerosis, cortical malformations, mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS), gelastic seizures with hypothalmic hamartoma | **Symptomatic:** *secondary to a known or presumed disorder of the brain* |
| **Metabolic:** *caused by a metabolic disorder of the brain* | *Pyroxidine deficiency, GLUT1 deficiency , etc* | **Symptomatic** |
| **Immune:** *epilepsy with evidence of autoimmune mediated CNS inflammation* | *NMDA receptor antibody encephalitis, voltage gated potassium channel antibody encephalitis* | **Symptomatic** |
| **Infectious**: *an infectious etiology refers to a patient with epilepsy, rather than seizures occurring in the setting of acute infection such as meningitis or encephalitis. These infections sometimes have a structural correlate.* | *Tuberculosis, HIV, cerebral malaria, neurocysticerosis, subacute sclerosing panencephalitis, cerebral toxoplasmosis* | |
| **Unknown**: *the cause of epilepsy is unknown* | | **Cryptogenic:** *presumed symptomatic* |

| Terminology | Terms no longer recommended |
|---|---|
| **Self-limited:** *tendency to resolve spontaneously over time* | **Benign** |
| **Pharmacoresponsive:** *highly likely to be controlled with medication* | **Catastrophic** |
| **Focal seizures:** *seizure semiology described according to specific subjective (auras), motor, autonomic, and dyscognitive features* | **Complex Partial** |
| **Evolving to a bilateral convulsive seizure** | **Simple Partial** |
| | **Secondary generalized** |

**We would welcome your thoughts on this proposal. Please visit the "Request for Comments" page on the ILAE website to read the full document and register your comments.**

**http://www.ilae.org/Visitors/Centre/Organization.cfm**

**References:** 1.Berg AT et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia 2010;51:676-685. 2.Blume WT et al. Glossary of descriptive terminology for ictal semiology: Report of the ILAE task force on classification and terminology. Epilepsia 2001:42;1212-1218. 3. Scheffer IE et al. The Organisation of the Epilepsies: Report of the ILAE Commission on Classification and Terminology (ILAE website as above)

In the upper panel classification of the seizures are given, in the middle panel the epileptic syndromes and other epilepsies grouped by specificity of diagnosis are given, in the lower panel the major changes in terminology and concepts are shown.

## Absence Epilepsy

Poupart introduced "absence" as a term to describe seizures in 1705, which was followed by the terms "petit mal" in 1838, and "pyknolepsy" in 1916 (Loiseau, 2002). In 1981, the ILAE classified absence seizures in the generalized seizures category (see the 2011-2013 classification Table 1), with electroclinical characteristics which they later defined, differentiating between typical and atypical absence seizures (Loiseau, 1992).

Typical absence seizures (TAS) are accompanied by a highly characteristic pattern of brain activity, so called spike and wave discharges (SWDs), which can be recorded in the EEG of the patients. During these typical absence seizures the EEG shows generalized, regular and synchronous SWDs with a frequency of 3 Hz (Bosnyakova et al., 2007). They usually start with a slightly higher frequency of 4-5 Hz and gradually decrease to a frequency of 2.5 Hz at the end of the SWDs. The clinical phenotype is the marked alteration of consciousness, and it might be connected by automatisms if the episode is long (old concepts of simple and complex absence seizures), and occasionally also by slight myoclonus (ILAE, 1981; Onat et al., 2013). Atypical absence seizures epilepsy (AAS) are clinically distinct from typical absence seizures (Carmant et al., 1996) for the following reasons: (1) the SWDs frequency is less than 3 Hz, (2) there is voluntary movement and partial consciousness during the seizures, (3) they are accompanied by severe cognitive and neurodevelopmental impairments, and (4) they are often intractable to antiepileptic drugs (Nolan et al., 2005). Two more absence seizure types with specific features were identified by the ILAE in 2010 (Berg et al., 2010). In myoclonic absence seizures the EEG is similar to that of typical absence seizures, but the now seizures are accompanied by marked bilateral rhythmical clonic jerks. In eyelid myoclonia absence seizures, impairment of consciousness is bland and EEG shows rhythmic diffuse polyspike-wave discharges, normally at 5–6 Hz (Weiergräber et al., 2010).

Above the seizures were described. Now the disease at which the seizures are seen are described. Childhood absence epilepsy (CAE) accounts for 2% to 8% of patients with epilepsy, with the main cause being predominantly genetic. About one-third of the families of children with CAE report a family history of similar seizures. The siblings of children with CAE have about a 10% chance of developing epilepsy. CAE is considered to be one of the relatively benign childhood epilepsies. For 65% of these children, the seizures respond to treatment and disappear. A recent investigation showed that children who initially obtained the first-line medication, ethosuximide (ESM), were less likely to experience subsequent relapses for any reason, when compared to children who were given valproic acid (VPA). After ≥5 years of follow-up, children who were initially treated with ESM were more likely to be seizure free for at least 5 years at their final follow-up. More importantly, they were much more likely to be in total remission (both 5 years seizure free and medication free).

The seizures of CAE normally begin between the ages of 4 and 8 in children who have normal intelligence and neurologic functions (Loiseau et al., 2002). It seems that

during these seizures the child loses the ability to maintain contact with the environment (van Luijtelaar et al., 1991), the child's eyes may roll up briefly, occasionally with some mild, myoclonic twitches of the facial muscles, and often not able to respond to external stimuli like a question addressed to them by their parent or teacher (Gloor, 1986). Often the child is not even aware that anything has happened. These episodes may occur 1 to 50 times per day.

Tonic-clonic seizures, with or without fever, might occur for a while before absence seizures develop and, from time to time, thereafter. Absence seizures persisting into adult life are rare, but they may happen sometimes. The presence of the SWDs in the EEG is the major diagnostic criterion for CAE, given the non-convulsive nature of this syndrome. As reviewed above, it must be noted, however, that there are also some other epileptic syndromes, i.e. juvenile absence epilepsy, juvenile myoclonic epilepsy, myoclonic absence epilepsy and eyelid myoclonia with absences, in which SWDs can be detected (Panayaiotopoulos, 1999).

# Therapeutics for Epilepsy

## Anti-seizure drugs

There are a wide variety of drugs on the market today that treat the seizures themselves, that is, they reduce the probability that seizures occur (Kaminski et al., 2014), they are named antiseizure drugs, although the name anti-epileptic drugs is commonly used as well.

Anticonvulsants are the first line treatment option for those who have a convulsive seizure disorder. The mechanism of action of these drugs can vary significantly (for review see Löscher et al., 2013), but for those patients who respond well, an oral anticonvulsant may be the only necessary medical intervention for the disorder.

Anti-absence medications are similar to anticonvulsants, in that they treat the seizures themselves, but the mechanism of action of these medications is often quite different. It so happens that most typical anticonvulsants such as phenytoin, carbamazepine and tiagabine actually exacerbate non-convulsive seizures, and conversely, some medications that are used to treat non-convulsive seizures such as ESM are ineffective against convulsive seizures (Panayiotopoulos, 1999; Rogawski and Löscher, 2004).

ESM (Table 2) is one of the most aged anti-absence drugs, next trimethadione, which is not longer used because of its toxicity. Although ESM's action mechanism is not completely understood, the blockade of a specific voltage-gated calcium channel (the T-type channel) in thalamic neurons appears central to its anti-seizure activity. This blockade suppresses thalamic excitability, which is believed to be necessary in order to sustain the characteristic SWDs of absence seizures (Huguenard et al., 2002). Serious adverse events that are connected to ESM include aplastic anemia, pancytopenia, Ste-

vens-Johnson syndrome (SJS), systemic lupus erythematosus and effects on the digestive system (Panayiotopoulos, 2001 Tenney and Jain, 2014). There are no published evidences on ESM-caused absences exacerbation. ESM's main impediment is the drug's ineffectiveness against other seizures types, especially tonic-clonic seizures (Panayiotopoulos, 2001).

**Table 2** From Vrielynck, 2013.

Summary of AED efficacy in different absence seizure types

| | TAS | AAS | EMA | MA | Main advantage | Main disadvantage |
|---|---|---|---|---|---|---|
| Valproate | +++ | ++ | ++ | ++ | Effective against all seizure types | Should be avoided in girls of childbearing age |
| Ethosuximide | +++ | ++ | +? | ++ | Minimal cognitive side effects | ineffective against tonic-clonic seizures |
| Lamotrigine | ++ | + | +? | +? | Favorable tolerability profile | Long dose titration |
| Levetiracetam | + | ? | ++ | ? | Favorable tolerability profile | |
| Rufinamide | ? | ++ | ? | ++? | Effective against drop-attacks in LGS | Limited experience |
| Benzodiazepine | ++ | ++ (CLB) | ++ (CNZ) | ? | Rapidly effective | Risk of dependence and habituation |

**Notes:** Degree of efficacy: +++ high, ++ moderate, + weak, ? unknown.

**Abbreviations:** TAS, typical absence seizures; AAS, atypical absence seizures; EMA, eyelid myoclonia with absences; MA, myoclonic absences; CLB, clobazam, CNZ, clonazepam; LGS, Lennox-Gastaut Syndrome; AED, antiepileptic drug.

## Anti-epileptogenic drugs

The ultimate goal of any drug therapy is to cure the patient of illness; whether the patient is then able to stop taking the medication may also be part of that goal.

A recent study showed that children who initially received ESM, still and for a long time it is considered as a first line medication, were less likely to experience subsequent relapses for any reasons compared to children who received VPA. After ≥5 years of follow-up, children initially treated with ESM were more likely to be at least 5-years seizure free at their last follow-up. Specifically, they were much more likely to be in complete remission (both 5-years seizure free and medication free). The difference appeared present even for 10-year remission. These findings are in line with those of studies in rat models of absence epilepsy (see below) and suggest potential disease modifying effects of ESM in childhood absence epilepsy (Berg et al., 2014).

Until now, it seems that our medical establishment is always a step behind illness, treating symptoms as they occur as opposed to treating the underlying cause of the illness or even better, being able to predict a particular illness and prevent it from occurring. This would be the goal of antiepileptogenesis; preventing the development of epilepsy in people who would otherwise have it. For example, it is known there is a likelihood of developing a seizure disorder in people who have had a traumatic brain injury (TBI, (Pitkänen and Bolkvadze, 2012)). If those people were given some treatment or compound that would prevent the neuropathological changes that lead to the seizure

disorder, this treatment would be called an antiepileptogenic (Löscher and Brandt, 2009). Such prevention treatment is not yet available for humans, although studies in animal models with ESM (Blumenfeld et al., 2008; van Luijtelaar et al., 2013), vigabatrin (Russo et al., 2011), levetiracetam (Kaminski et al., 2014) and cannabinoids (Vinogradova et al., 2015) are encouraging for future development of drugs preventing the development of epilepsy.

# Rodent Models of Epilepsy

## Animal Models: Necessary and Valid

Animal models are necessary to investigate and understand basic principles of various diseases. Beyond the necessity of their use, epilepsy models used must be proven valid; this means that various conditions must be met. For example, the seizure model must correctly predict the response of standard drug and experimental treatments. Also, the model must reflect some of neuropathological changes that occur in the human condition. Finally, the overt behavior of the animal must mirror those seen in humans with the disease.

## Genetic Models for epilepsy

One of the greatest advantages of using mice in research is that the genetic sequence of the animal is well characterized. If one gene or a cluster of genes is thought responsible for a condition, it is relatively easy to manipulate that and create a sub-species knockout line wherein all of the offspring carry the deleted, silenced, or in some way mutated and quiescent version of the gene.

Animal mutants or transgenic animals with spontaneously recurrent seizures are some examples of true models of epilepsy, which are obviously more closely related to human epilepsy than simple seizure models (Fig. 1). Unfortunately, researchers usually do not make a differentiation between animal models of epilepsy and animal models of epileptic seizures, in spite of this important difference when interpreting data obtained with such models. Models of epilepsy, e.g. mutant animals with inherent epilepsy, can of course be used as models of seizures, such as in anti-seizure drug potency studies. However, a pure seizure model in a non-epileptic animal is not applicable as a chronic epilepsy model.

The focus of this thesis is absence epilepsy: therefore the existing absence seizure models and absence epilepsy models will be introduced. A few models for other types of seizures and epilepsy will be briefly mentioned since some of the drug studies that will be discussed in the introduction and general discussion of this thesis, have used these animal models. First the genetic absence epilepsy mouse models will be described, such as lethargic, stargazer, and tottering mice (Fig. 1). Next, rats with a genetic form of absence

epilepsy such as WAG/Rij rats and GAERS, and finally induced non-absence models such as DBA mice and the pilocarpine SE model.

## Mouse models related to absence epilepsy

### Lethargic mice

Mice of the lethargic (lh/lh) strain have an inherited mutation of the calcium channel β4 subunit (Fig. 1). This mutation truncates the C-terminus of the β subunit of the P/Q type calcium channel such that the binding site for α- subunit is missing (Burgess et al., 1999). This ultimately results in drastically altered calcium channel kinetics and the "lethargic" phenotype of the mice. Specific work has pointed to the ventrolateral and reticular nuclei of the thalamus as the generator network of the seizures in lh/lh mice (Hosford et al., 1992). Behaviorally, these mice are typically ataxic. When movements do occur, they are unstable, lurching and generally uncoordinated (Hosford and Wang, 1997). The EEG of lh/lh mice shows periodic, spontaneous SWDs that is typically 3 to 4 times the amplitude of the background EEG signal and falls within the 4 to 6 Hz frequency range (Hosford et al., 1992). Lethargic mice are known to respond well to the classic first-line therapeutic for absence epilepsy: ESM (Aizawa et al., 1997).

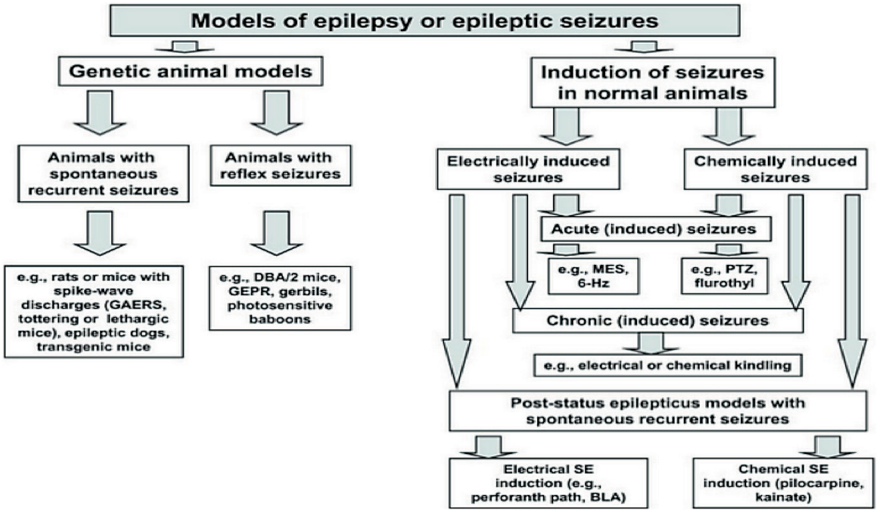



**Figure 1** An overview of models of epilepsy or epileptic seizures. Note that there are numerous models not shown in this figure, including chronic epilepsy models, in which spontaneous recurrent seizures develop after traumatic brain injury, ischemic brain damage, or febrile seizures. For more details see Löscher and Pitkänen et al., 2006.

**Stargazer and tottering models**

Other mouse models of genetic absence epilepsy include the stargazer and tottering models, among others (Sarkisian, 2001). These mice are quite similar to the lh/lh model in terms of behavioral phenotype. Stargazer mice also have an inherited mutation in the calcium channel regulating genes, although in this case it is in the gamma subunit (Osten and Stern-Bach, 2006). The tottering mouse, with a quite pronounced absence-like phenotype and motor dystonia, has a calcium channel dysfunction similar to that of the lethargic mouse (Fletcher et al., 1996; Campbell and Hess, 1999).

**Other mutant mice models**

Abnormalities in excitatory and inhibitory neurotransmission in the cortico-thalamic network underlying absence seizures appear in mice with the genetic deletion of mGlu4 receptors (Wang et al., 2005). In a recent study, mice with gene targeted deletion of mGlu4 showed a significantly larger susceptibility to pilocarpine-induced acute convulsive epileptic seizures (Pitsch et al., 2007). However, these mice are resistant to chemically induced absence seizures (Snead et al., 2000). In line, pharmacological and gene targeting studies have provided evidence that the inhibition or absence of mGlu4 receptors render rodents resistant to absence epilepsy (Snead et al., 2000). It is suggested that transcriptional regulation of mGlu4 expression occurs in response to convulsive seizures as a compensatory mechanism to counterweight changes in glutamatergic or GABAergic activity (Sperk et al., 2013). Normal mice are indeed protected against pentylenetetrazol-evoked absence seizures when bilaterally injected with an mGlu4 receptor antagonist in the thalamus (Snead et al., 2000). It then must be investigated whether changes in the expression/activity of mGlu4 receptors within the cortico-thalamo-cortical circuitry are in some way correlated with the occurrence of spontaneous absence seizures.

## Rat absence epilepsy models

After the discovery of the genetic rat models (Fig. 1), the mice models and the chemical absence models were less often used. Two rat-models are leading in the literature: Genetic Absence Epilepsy Rats from Strasbourg (GAERS) and Wistar Albino Glaxo rats from Rijswijk (WAG/Rij) (Fig. 2), both exhibit SWDs in the 7 to 11 Hz frequency range (van Luijtelaar and Coenen, 1986; Charpier et al., 1999; Polack et al., 2007. Coenen and van Luijtelaar, 2003, van Luijtelaar and Sitnikova, 2006). Both the GAERS and WAG/Rij rodents meet the criteria to be considered as valid models of absence epilepsy: the behavior of the epileptic animal is similar to epileptic humans, the neuropathology is similar and the response to the first line antiepileptic medication is similar (Coenen and van Luijtelaar, 2003; Depaulis and van Luijtelaar, 2006). Indeed, both types of rats exhibit behavioral arrest and impaired consciousness during the synchronous and bilateral EEG events, the SWDs (Marescaux et al., 1992; Coenen and van Luijtelaar, 2003; van Luijtelaar and Sitnikova, 2006). The EEG waveforms of the SWDs is strikingly similar to the ictal waveform seen in human patients

(Sitnikova and van Luijtelaar, 2007), although the frequency of the oscillations is higher in rodents than in patients (7-11 Hz in rodents, versus 3 Hz in humans)(van Luijtelaar and Coenen, 1986; Benbadis et al., 2001).

## Characteristics of SWDs in rat models of absence epilepsy

The spontaneous occurrence of SWDs in the cortical EEG does not only apply to GAERS and WAG/Rij rats. There are various types of other laboratory animals that have been indicated as having spontaneous SWDs (Chocholova, 1983; Klingberg and Pickenhain, 1968; Buzsaki et al., 1990a; Buzsaki et al., 1990b; Inoue et al., 1990; Willoughby and Mackenzie, 1992; Shaw, 2004;), including e.g. the tottering mouse and lethargic mouse. However, it must be noted, that some of these animals also show other neurological disturbances next to SWDs (Crunelli and Leresche, 2002; Paz et al., 2011).

A single gene may be responsible for the presence of SWDs, which has been shown in quantitative genetic studies in GAERS (Rudolf et al., 2004) and WAG/Rij rats. However, the amount of seizures is determined by only a few other genes (Peeters et al., 1992). SWDs are the same in both genders both in GAERS and WAG/Rij rats (Coenen and van Luijtelaar, 1987; van Luijtelaar et al., 2014), indicating that the genetic transmission is autosomal. SWDs in WAG/Rij rats are first detected around 60–80 days of age (Coenen and van Luijtelaar, 1987; Schridde and van Luijtelaar, 2004a; Schridde and van Luijtelaar, 2004b; Schridde and van Luijtelaar, 2005). The first SWDs are few (1 or 2 hours) and short-lasting (1–3s) at that age, the wave is not developed yet, the inter-spike frequency during a discharge is also lower (4–5Hz), the spikes are not very sharp, and the pattern of SWDs is less ordered and exact. With age, the number, duration, morphology and

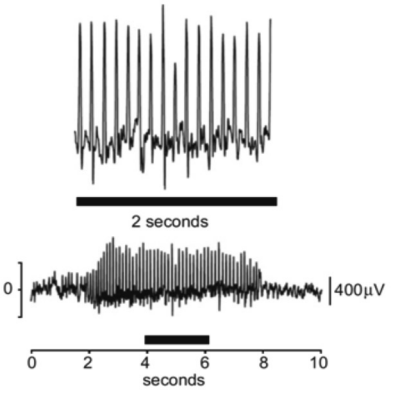



**Figure 2** Photograph of a WAG/Rij rat implanted with electrode for ECoG recording: Spontaneous incidence of SWDs: 16-20/hour - Mean duration: 5 seconds.
(Photo Herbert van der Sluis).

frequency of SWDs increases, whereas their amplitude is not modified. The number of animals also increases with SWDs depending on age: 50% of the WAG/Rij rats display fully developed SWDs at 3 months of age, and 100% of the animals show mature SWDs (about 16–20 per hour) at 6 months of age, with a frequency of approximately 7-11 Hz, and a mean duration of about 5s (Coenen and van Luijtelaar, 1987; Chahboune et al., 2009). After 6 months, there is still an age-dependent enhance in the number of SWDs and in the mean duration. The SWDs do not change to other type of seizures or spontaneously remit.

## The cortico-thalamic-cortical network

Disturbances in glutamatergic or GABAergic neurotransmission are among the causes responsible for the origin and spread of SWDs, characterizing absence epilepsy. The SWDs, the electroencephalographic hallmark of absence seizures, start in the deep layers of the somatosensory cortex and quickly spread over the cortex and to the cortico-thalamo-cortical (C-T-C) circuitry (Meeren et al., 2002; Blumenfeld et al., 2005; Polack et al., 2007). This circuitry consists of glutamatergic projections from the deep layer cortical neurons in the somatosensory cortex (S1po) to ventrobasal (VB) thalamic nuclei including the posterior nucleus PO and to the GABA-ergic reticular thalamic nucleus (nRT); glutamatergic projections from thalamic nuclei to the cortex and nRT and GABA-ergic projections from the nRT to the thalamic nuclei. These latter projections are responsible for widespread thalamic inhibition (Blumenfeld et al., 2005; van Luijtelaar and Sitnikova, 2006).

## Response to antiepileptic drugs in WAG/Rij rat and GAERS

GAERS and/or WAG/Rij rats have been tested on with the well-known classical antiepileptic drugs in order to establish the pharmacological validity of both models. The four main AEDs which are effective against human absences suppress the SWDs: the typical anti-absence drugs ESM, the broad-spectrum drug VPA, the non-prescribed trimethadione, and, in principle, all benzodiazepines (van Luijtelaar et al., 2002; Manning et al., 2003). Contrastingly, SWDs are worsened by drugs that are either ineffective or aggravate SWDs in humans (such as carbamazepine, phenytoin, and locasemide, all sodium channel blockers). The results of pharmacological tests in GAERS and WAG/Rij rats therefore show great similarities to the pharmacological reactivity of typical absence seizures in humans (Panayiotopoulos, 1999; Glauser et al., 2010). Pentylenetetrazol or penicillin—weak GABAA antagonists known to induce "absence-like" discharges in normal rats—aggravate SWDs in GAERS (Depaulis and van Luijtelaar, 2006).

## Induced models for epilepsy

Beyond the various genetic models of epilepsy, other models for the study of epilepsy exist that involve the induction of seizures using an exogenous compound or stimulus. Genetic models may be thought to be superior and somehow "more valid" because of similarities in the pathology involved in the human and rodent epilepsies. However,

induced models of epilepsy are fast, reproducible and often repeatable within subjects, making them an appealing option for investigation. However, it should be highlighted that genetic models do not exist for most of the epilepsies. In fact, this is the reason why the pharmacologically induced or chemical models for other types of seizures or epilepsy are very necessary. Moreover, the induced models have also been used to evaluate effects of classic AED and also new compounds.

## Induction of seizures by stimulation

### DBA mice

One of the most serious concerns for patients with epilepsy and their families is sudden unexpected death in epilepsy (SUDEP). The 21-day-old DBA mice (Fig. 1 ), have generalized convulsions when exposed to intense auditory stimulation. Furthermore, DBA mice exhibit SUDEP in the phase immediately following audiogenic seizure (AGS), and respiratory arrest is the cause of death for these mice (RA) (Venit et al., 2004; De Sarro et al., 2015). If resuscitation of the mice was administered quickly, death could be avoided (Collins, 1972). When the DBA mice's oxygen levels were increased in the local environment, incidences of RA were reduced, and a greater percentage of these mice survived (Willott et al., 1976). This model has been retained in subsequent steps of preclinical AED development. DBA mice are useful models in the steps to clarifying the potency and spectrum of anticonvulsant activities that may be used to ward off different types of epileptic seizures. Clinical antiepileptic drugs such as diazepam, carbamazepine and VPA show therapeutic indications in DBA/2 mouse (De Sarro et al., 1996; 2000; 2015).

## Induction of seizures by exogenous compound

### Pilocarpine model

Multiple species, including rats and mice (Cavalheiro et al., 1996; Curia et al., 2008) and some non-human primates (Perez-Mendes et al., 2011) injected with the muscarinic agonist pilocarpine show generalized, convulsive seizures. The pilocarpine model (Fig. 1) is a model of both status epilepticus (SE, continuous seizure activity) and mesial temporal lobe epilepsy (Cavalheiro et al., 1996; Curia et al., 2008). Once pilocarpine is given, a period of SE develops wherein the animal exhibits more or less continuous behavioral and electrographic seizures that last for at least 30 minutes (Cavalheiro et al., 1996; Curia et al., 2008). There are bouts of clonic/tonic seizures that occur during this time, and it has been found that SE precedes a profound increase in glutamate release in the hippocampus, likely contributing the neurotoxicity of the model (Costa et al., 2004). Following the initial bout of SE, the animal goes through a silent, or latent, period during which no behavioral seizures occur, but significant neurological changes are occurring that allow for the eventual development of spontaneous, recurrent seizures (Cavalheiro et al., 1996; Estrada et al., 2012).

**Pentylenetetrazol model**

PTZ is a convulsive agent, which is generally used to different forms of experimental epilepsy models. It is a selective $GABA_A$ receptor channel blocker and is related with a decrease of GABA- mediated neurotransmission (Bambal et al., 2011). Systemic injection of the $GABA_A$ antagonist PTZ induces primary generalized seizures. In small doses, PTZ has been used as a model to induce absence seizures. In wild-type mice small doses of PTZ (30 mg/kg, s.c.), induced absence-like seizures characterized by bilaterally synchronous SWDs. The letter was also found in rat models (Snead OC 3rd, 1992). A higher PTZ dose arise convulsive seizures, and together with the Maximal Electroshock model (MES), one of the most commonly used models for antiepileptic drug assessment (Pitkänen, 2006; Löscher, 1982). A behavioural scoring system for PTZ induced non-convulsive and convulsive seizures has been described (Lüttjohann et al., 2009).

## Ionotropic glutamate receptors

Ionotropic receptors are transmembrane molecules that are able to "open" or "close" a channel. This allows smaller particles to travel in and out of the cell. As the name suggests, ionotropic receptors permit various types of ions to enter or leave nerve cells via transport molecules in the cell membrane. Ionotropic receptors are neither always opened nor closed. They tend to be closed until another small molecule (called a ligand – in our case, a neurotransmitter) binds itself to the receptor. As soon as the ligand binds to the receptor, the receptor alters its conformation (the protein that forms the channel changes its shape). As this occurs, a small opening is created that is just big enough for ions to travel through.

Glutamate (and aspartate or other excitatory endogenous compounds such as quinolinate (QUIN) or some sulfur-containing amino acids, in addition to more potent selective agonists including NMDA, AMPA, kainate, ibotenic acid and domoic acid) can cause convulsions when focally or systemically administered to experimental animals. Glutamate exerts its excitatory action by way of ligand-gated ion channels (NMDA and non-NMDA receptors) to raise sodium and calcium conductance. Innumerable reciprocal regulatory interactions occur between the activation of glutamatergic receptors and other transmitter systems, ion transport, gene activation and receptor modification. The flexibility and intricacy of these interactions puts glutamate-mediated transmission in a crucial position for tempering the excitatory threshold of pathways that are involved in the generation of seizures (Moldrich et al., 2003).

The anticonvulsant properties of ionotropic glutamate receptor antagonists have been scrupulously reviewed (Chapman, 1995; Meldrum and Chapman 1999a; Rogawski, 1992). All classes of NMDA receptor antagonists (competitive NMDA antagonists, channel site antagonists, glycine site antagonists, polyamine site antagonists), in addition to competitive and noncompetitive AMPA/kainate antagonists, exhibit wide-spectrum anticonvulsant properties in both acute and chronic animal epilepsy models. These are

associated with varying degrees of behavioral side effects, which can include from minimal for some of the glycine site or competitive NMDA antagonists, to extensive for some of the high-affinity NMDA antagonists.

Moreover, it has been showed that injection of noncompetitive NMDA, AMPA receptor agonists, developed a fast dose-dependent increase in the number of SWD in WAG/Rij rats (Peeters et al., 1994a). And indeed noncompetitive NMDA, antagonist caused a dose-dependent reduction in the number of SWDs in the same model (Peeters et al., 1990; Peeters et al., 1994b).

## Metabotropic glutamate receptors

MGlu receptors make up a family of eight subtypes that are subdivided into three groups based on amino acid sequence, pharmacologic profile, and G-protein coupling (Conn and Pin, 1997). As can be seen in Fig. 3 A, group I is comprised of mGlu1 and mGlu5 receptors, which are coupled to Gq proteins. Their activation encourages polyphosphoinositide hydrolysis to form inositol-1,4,5-trisphosphate and diacylglycerol. mGlu1 and mGlu5 receptors also control the activity within various types of calcium and potassium channels (Hermans and Challiss, 2001). Group II receptors (mGlu2 and mGlu3 respectively), which are coupled to Gi/Go proteins and negatively modulate the activity of adenyl cyclase and voltage-sensitive calcium channels (VSCCs). Group III (mGlu4, mGlu6, mGlu7, and mGlu8 receptors), which are also coupled to Gi/Go proteins (Conn and Pin, 1997) (Fig. 3 A).

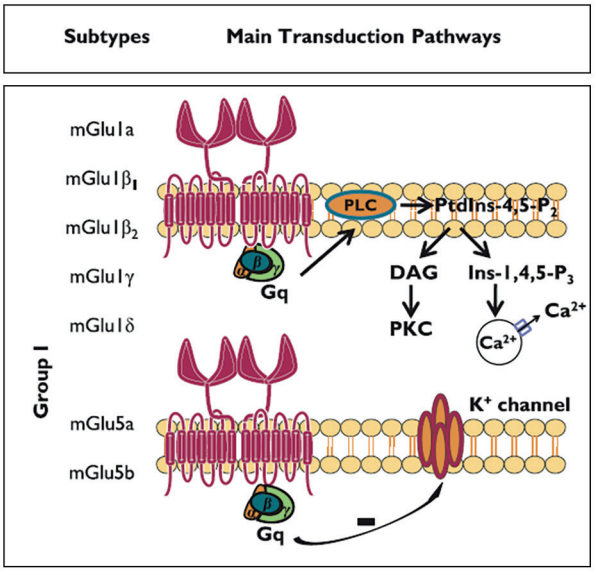



**Figure 3A** mGlu Receptors Group I subtypes, classification, signaling and pathway.

## Localization of mGlu receptors in the network of absence seizures

mGlu receptors are located at synapses of the C-T-C network, which arise SWDs connected to absence seizures. This indicates that these receptors are possible targets for the treatment of absence epilepsy (Ngomba et al., 2011). Group I mGlu receptors are preferentially expressed in the peripheral portion of the postsynaptic density, where they arise excitatory responses and control mechanisms of synaptic plasticity. mGlu1 and mGlu5 receptors are also express postsynaptically on neurons of ventrobasal (VB) thalamus. Thalamic relay neurons in particular display a high density on their dendrites of two isoforms of mGlu1 receptors, specifically mGlu1a and mGlu1c receptors (Ferraguti et al., 2008), postsynaptic to axon terminals originating from cortical layer VI neurons (Martin et al., 1992; Shigemoto et al., 1992; Baude et al., 1993; Godwin et al., 1996; Vidnyanszky et al., 1996; Liu et al., 1998).

On the other hand, mGlu2, mGlu3, mGlu4, mGlu7, and mGlu8 receptors occur preferentially (though not exclusively) in presynaptic terminals, where they negatively control neurotransmitter release (Ferraguti and Shigemoto, 2006). mGlu6 Receptors exist almost exclusively on retinal ON-bipolar cells, and therefore do not play a direct role in the C-T-C circuits (Pin and Duvoisin, 1995; Morgans et al., 2010) (Fig 3 B).

## Electrophysiology of group I mGlu receptors in the network of absence seizures

The neocortex and thalamus have been extensively reviewed on their circuitry and the network properties of the connections between them (McCormick and Bal, 1997; Sherman and Guillery, 2002; Alexander and Godwin, 2006a). The firing patterns of thalamic neurons are contingent on the complex interaction between a number of fundamental conductances of thalamic relay cells and interneurons (McCormick and Bal, 1997; Crunelli et al., 2002).

The group I receptors' role in modulating the excitability of thalamic relay cells has been well characterized on the basis of studies done on rodents and non rodents in numerous relay nuclei (Salt and Eaton, 1996; Alexander and Godwin, 2006a). It appears that under physiologic conditions these receptors take part in sensory processing by acting as mediators for cortical inputs back into the thalamus (Salt and Turner, 1998a; Rivadulla et al., 2002). Repetitive, high-frequency stimulation of cortico-thalamic afferents that are thought to arise from layer VI of the cortex can synaptically activate these mGlu1 receptors (Hughes et al., 2002; Reichova and Sherman, 2004), and they can control the oscillatory properties of thalamic relay cells (Crunelli et al., 2002; Hughes et al., 2002).

The hypersynchronous oscillatory activity of thalamic relay neurons underlying SWDs is prolonged by the activation of T-type VSCCs, which recovers from inhibition in the hyperpolarizing environment created by the GABAergic input from nRT projection neurons (reviewed by Crunelli and Leresche, 2002; Blumenfeld, 2005). More specifically, the activation of mGlu1 receptors leads to an intrinsic slow (<1 Hz) oscillation in thalamic relay neurons, which suggests there is a role for these receptors in the production of

slow-wave sleep rhythms (Hughes et al., 2002). This mechanism relies on the "window" component of the T-type calcium channels - $Ca^{2+}$-current and a $Ca^{2+}$-activated -, nonselective cation current (Hughes et al., 2002). mGlu5 receptors in the thalamus have an excitatory effect comparable to that mediated by mGlu1 receptors. Consequently, they can partake in sensory responses of thalamic relay cells (Salt and Binns, 2000).

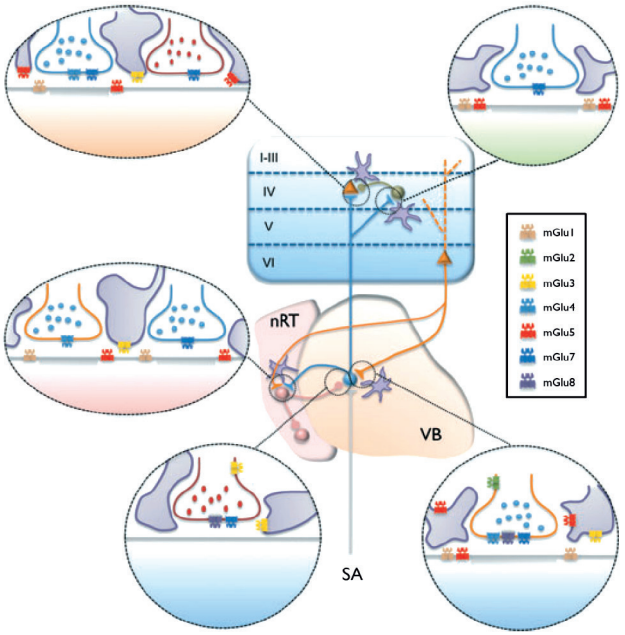



**Figure 3B** Schematic diagram of the cortico-thalamo-cortical loop highlighting the synaptic distribution of distinct mGlu receptor subtypes. Thalamic relay neurons (light blue) in the ventrobasal thalamus (VB) send glutamatergic projections (triangular endings) to layer IV pyramidal neurons (orange) and interneurons (green) of the neocortex (blue box). In turn, pyramidal neurons in layer VI send topographically organized glutamatergic afferents back to thalamic relay neurons, and to neurons of the reticular thalamic nucleus (nRT, pink). Neurons of the nRT send GABAergic projections (round endings) to relay neurons and local collaterals to other nRT neurons. Most of these synapses are ensheathed by processes of astrocytes (purple). Sensory afferents (SA) to relay neurons are exemplified by the gray line. The subcellular distribution of distinct mGlu receptors (displayed in different colors, as shown in the box) is shown for specific synapses (dotted circles). Glutamatergic terminals are shown with blue lines and vesicles, whereas GABAergic terminals are depicted with red lines and vesicles. Astrocytic processes are in purple. A thicker gray line in the postsynaptic element defines the postsynaptic specialization of asymmetric synapses. mGlu1 and mGlu5 receptors are mostly present perisynaptically at asymmetrical synapses (Ngomba et al., 2011).

## Orthosteric vs. allosteric modulators

Various compounds have been well-developed (Table 3) that specifically target singular mGlu receptor subtypes or groups of subtypes. Orthosteric agonists or antagonists interact with the glutamate binding site expressed in the N-terminal domain of mGlu receptors, while the binding sites for positive and negative allosteric modulator (PAMs and NAMs) are most often expressed in the 7-TM domain (reviewed by Pin et al., 2005). By exclusively recruiting receptors activated by an endogenous ligand, such as glutamate, PAMs amplify the receptor's function in the presence of an orthosteric agonist. Furthermore, these compounds may preserve the standard role of endogenous neurotransmitters. This is specifically true for PAMs, which need the regular release of the endogenous neurotransmitters on the G protein-coupled receptor (GPCR). They also enhance neurotransmitter action in a more physiological suitable manner. Therefore, preserving the natural mechanisms that regulate synaptic function may elicit fewer adverse effects and greater efficacy than would be the case with a traditional agonist, which bypasses normal mechanisms for regulating synaptic transmission (Wootten et al., 2013) (Fig. 4).

For example, mGluR5 plays a considerable function in two opposing forms of synaptic plasticity in the hippocampus. These two are included by highly specific patterns of activity of glutamatergic afferents and are known as long-term potentiation (LTD) and long-term depression (LTD) of synaptic strength (Mukherjee and Manahan-Vaughan, 2013). In fact, mGluR5 orthosteric agonists provoke profound LTD at the cost of LTP (Gasparini et al., 1999; Huber et al., 2001). This shift in the balance of LTP and LTD arises with enhanced mGluR5 function in fragile X syndrome (FXS) and is believed to lead to cognitive disruption in patients with FXS (Bear et al., 2004). Therefore, mGluR5 activation with a PAM could improve both forms of synaptic plasticity at the same time, thereby enhancing cognitive function (Noetzel et al., 2012; Ayala et al., 2009). This creates an ideal profile for increasing cognitive function, and mGluR5 PAMs certainly enhance cognitive function in multiple rodent models (Chan et al., 2008; Gastambide et al., 2013).

Remarkable is that, depending on the synapse-specific modulatory function of mGlu receptors, one is able to predict a better profile of safety and tolerability of the mGlu allosteric modulators, when compared to ionotropic (AMPA or NMDA) receptor antagonists (see Bruno et al., 2001). The discoveries of the mGluR1 NAM CPCOOEt (Litschig et al., 1999) and the mGluR5 NAM SIB-1757 (Varney et al., 1999) uncovered the first highly selective antagonists for any individual mGluR subtype. These developments paved the way for intense investigations that have yielded highly selective NAMS and PAMs for most of the eight mGluR subtypes (O'Brien et al., 2004; Niswender et al., 2008; Urwyler, 2011; Byun et al., 2014). Some of these have already been evaluated in epilepsy and seizure models.

## Effects of mGlu ligands in animal models of convulsive epilepsies

### DBA/2 mice

Older studies with two non-selective Group I mGluR antagonists ((S)-4-carboxy-3-hydroxyphenylglycine and (S)-4-carboxyphenylglycine) first demonstated a role for mGluR in epilepsy and that compounds of this group may be anticonvulsant (Thomsen et al., 1994; Dalby and Thomsen, 1996).

- Group I mGluR antagonists (phenylglycine-like) have potent anticonvulsant activity.

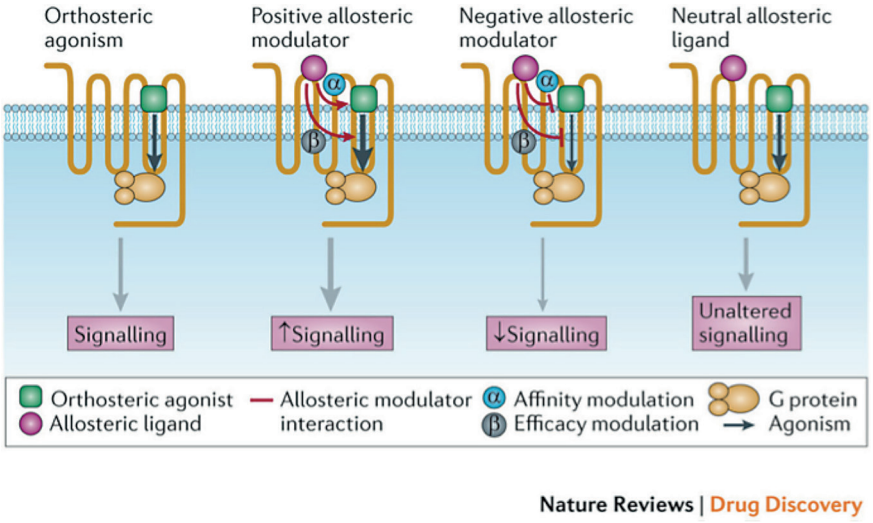



**Figure 4** Orthosteric agonists bind to the G protein-coupled receptor (GPCR), which induces a conformational change that results in the activation of downstream signaling. Positive allosteric modulators are allosteric ligands that bind to a topographically distinct site to the orthosteric agonist and enhance the affinity (cooperativity factor-α) and/or efficacy (modulation factor-β) of the orthosteric agonist. Negative allosteric modulators are allosteric ligands that decrease the affinity (cooperativity factor-α) and/or efficacy (modulation factor-β) of the orthosteric agonist. Allosteric ligands that have no effect on the affinity and/or efficacy mediated by the orthosteric agonist are termed neutral allosteric ligands. The red arrows denote the allosteric interaction of the modulator with the orthosteric ligand, and the black arrows denote the allosteric interaction between the ligand binding sites and the effector binding site within the GPCR, resulting in downstream activation of signaling pathways ((this is known as orthosteric agonism: Wootten, Christopoulos and Sexton, 2013)).

## Pentylenetetrazol model

Previous reports found that an intracerebroventricular (i.c.v.) injection of an antagonist mGlu1, AIDA, showed anti-absence effects in the PTZ model (Chapman et al., 1999; Tang et al., 2004). Low doses of PTZ induce typical changes in the EEG characterized by trains of SWDs at 5 ± 7 Hz with no clear overt behavioural response. LY367385 (mGluR1 antagonist) and 6-Methy l-2- (phenylethynyl) pyridinehydrochloride;2-methyl-6-(phenylethynyl) pyridine hydrochloride; MPEP (Hydrochloride; mGluR5 antagonist) were also used in order to investigate their effects on PTZ induced non-convulsive seizures (Table 3). Both drugs exhibited antiepileptic efficacy against seizures induced by low doses of PTZ, but they were not effective in opposing seizures provoked by higher PTZ doses (Nagaraja and Grecksch, 2004), suggesting an anti-absence effect but not an anticonvulsive effect.

SIB 1893 (a non-competitive antagonist of group I mGluR5) given at 40 mg/kg (but not at 20–30 mg/kg), failed to influence PTZ-caused convulsions in mouse (Borowicz et al., 2003, see Table 3). The selective group II mGluR agonist LY354740, administered prior to an injection of PTZ, generated a dose-dependent reduction in the number of mice showing clonic convulsions (Linden et al., 2002).

- Low dose PTZ is a model for absence seizures.
- Antagonist mGlu1 (AIDA, LY367385) and antagonist mGlu5 (MPEP) have anti-absence effects in low dose PTZ model.
- The non-competitive mGluR1 antagonist SIB 1893 failed to influence PTZ-induced convulsions in mice.
- The group II mGluR agonist LY354740 is an anticonvulsant drug as appeared from the high-dose PTZ model.

## Pilocarpine model

In the pilocarpine SE mouse model, Chen et al. (2005) displayed an enhanced expression of inhibitory mGluR4 and downregulation of excitatory mGluR1 in epileptic mice, as well as a reduction in the excitatory mGluR1 and 5 in wild-type C57BL/6 strain – this suggests differential expressions of group I and III mGluR in different groups of animals. The action of a Group 2 mGluR agonist, LY379268, was investigated in a series of experiments using the pilocarpine model. The results showed that treatment with LY379268 was effective at reducing both behavioral correlates and power in EEG bandwidths associated with the spontaneous occurring seizures (Caulder et al., 2013).

- The group II mGluR agonist LY379268 is an anticonvulsant in the pilocarpine mouse SE model.

## Effects of mGlu ligands in animal models of absence epilepsies

### Low dose pentylenetetrazol model

The effects of mGlu ligands in low dose PTZ model were already described above (see pg.30).

### Lethargic mice

**Group I**

Blockade with the orthosteric mGlu1 receptor antagonists, AIDA or LY367385, decreases the SWD occurrence (Burgess et al., 1997; Chapman et al., 1999;). A functional coupling between T-type VSCCs and mGlu1 receptors (Hildebrand et al., 2007; 2009) may be part of SWD regulation by mGlu1 receptors.

mGlu1 receptor blockade with the orthosteric antagonists, AIDA or LY367385, reduces the incidence of SWDs (Chapman et al., 1999). A functional coupling between mGlu1 receptors and T-type VSCCs (Hildebrand et al., 2007; 2009) might be involved in the regulation of SWDs by mGlu1 receptors. If so, this coupling might by affected by a mutated b4 subunit of T-type VSCCs, as occurs in lethargic mice (Burgess et al., 1999). Furthermore, the selective mGlu5 antagonists, MPEP, caused a marked reduction in the incidence of spontaneous SWDs (Ngomba et al., 2011). It has been suggested that MPEP and another much more selective mGluR5 antagonist and to a lesser degree, an agonist of mGluR4, SIB-1893, both in vitro and in vivo act as NMDA receptor antagonists at somewhat higher doses than are required for mGlu5 antagonism (O'Leary et al., 2000; Movsesyan et al., 2001). Activation of mGlu5 receptors enables the triggering of NMDA receptors, and vice versa (Salt and Binns, 2000; Bertaso et al., 2010). This interaction could explain the protective activity of MPEP, at least in lh/lh mice, because the NMDA receptor blockade may also decrease SWDs (Peeters et al., 1990; Coenen and van Luijtelaar, 2003). For an overview of the effects of group I ligands on the incidence of SWDs see Table 3.

- mGluR1 antagonist (AIDA or LY367385) and mGluR5 antagonist (MPEP) have an anti-absence action in the lethargic mice absence model.

**Group II**

The activity of mGlu2/3 receptor ligands is not uniform in different animal models of absence seizures. To illustrate, in lh/lh mice, activation of mGlu2/3 receptors with the selective group II mGlu receptor agonist, LY379268, minimized absence seizure occurrences (Moldrich et al., 2001) while in WAG/Rij rats, the same agonist increased the incidence of SWDs (Ngomba et al., 2005). These opposite results might be explained if it is assumed that mGlu2 and mGlu3 receptors have distinct roles in the pathophysiology of absence seizures, and they are differentially expressed in lethargic mice and WAG/Rij rats.

**Group III**

- The absence of optimal subtype selective ligands has hampered the study of the function of individual group III mGluRs.

## WAG/Rij rats

**Group I**

- The role of group I metabotropic receptor agonist/antagonist in the WAG/Rij absence model will be evaluated in this thesis.

**Group II**

Previously it was found that symptomatic 6-month-old WAG/Rij rats exhibited an enhanced expression of mGlu2/3 receptors in the ventrolateral area of the somatosensory cortex (Ngomba et al., 2005). This includes the putative SWDs' "triggering zone" (Meeren et al., 2002). There was also an increase in receptor expression in the ventrobasal (VB) thalamus and in the hippocampus, but not in the nRT.

LY379268, an orthosteric agonist, is almost inert at mGlu1, mGlu4, mGlu5 and mGlu7 receptors, and binds to mGlu8 receptors with micromolar affinity. By comparison, the orthosteric antagonist LY341495 binds all mGlu receptor subtypes at micromolar concentrations (reviewed by Johnson et al., 1999). It was found that the agonist LY379268 in WAG/Rij largely enhanced the number of SWDs (Ngomba et al., 2005). LY341495, a drug that behaves as a preferential antagonist of these receptors, decreased SWDs in WAG/Rij rats.

- The orthosteric agonist LY379268 has a pro-absence action.
- The orthosteric antagonist LY341495 has an anti-absence action.

**Group III**

WAG/Rij rats display an enhanced prevalence of pathological SWDs when treated with the selective mGlu4 receptor enhancer, N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) (Ngomba et al., 2008). The mGlu4 receptor gene in humans is localized at a susceptibility locus for juvenile myoclonic epilepsy (Izzi et al., 2003; Wong et al., 2001), which is characterized by absence seizures, as well as by myoclonus and tonic-clonic seizures. The mGlu7 receptor, another group-III mGlu receptor, is also related to the development of absence seizures: mGluR7 knockout mice develop absence seizures when mGlu7 receptors are unable to interact with PICK1 (protein interacting with C kinase 1) (Bertaso et al., 2008; Zhang et al., 2008). The same occurs with mice injected with a peptide that disturbs the interaction between PICK1 and mGlu7 receptors (Bertaso et al., 2008). Compounds for mGlu7 receptors have not yet been developed and this fact has limited studies on the function of mGlu7 receptors in animal models of absence epilepsy.

- The selective mGlu4 receptor enhancer PHCCC has a pro-absence action.

## Effects of group I mGlu receptor modulators in epilepsy

In Table 3 the pro- and anticonvulsant activity of group I modulators is shown. Several ligands have been developed which specifically target singular mGlu receptor subtypes or groups of subtypes. Orthosteric agonist of Group I are proconvulsive agents, while all orthosteric antagonist are both anticonvulsant as well as anti absence drugs. A charming approach may be to exploit the development of new ligands such as subtype-selective PAMs or NAMs, in order to establish the functions of mGlu receptors during activation by endogenous glutamate in a physiologic environment. First of all, based on the outcomes of Table 3, in which the action of Group I orthosteric agonists and antagonists is compared between convulsive and non-convulsive epilepsy, it is striking that group I antagonist have the same pharmacological epilepsy reducing effects in convulsive and in non-convulsive epilepsy. This agrees with the proconvulsive action of the orthosteric agonists, while their effects in non-convulsive epilepsy have not been assessed. As can be seen in Table 3, in lh/lh mice and in low dose PTZ, group I antagonists are anti-absence, thus, decreasing epilepsy. By extrapolating from this, we predict that a selective modulator such as a group I NAM might have an anti-absence and a selective PAM a pro-absence effect.

## Overview of all metabotropic compounds in two absence epilepsy models

An overview of different metabotropic compounds (agonist/antagonist) administered in lh/lh mice and WAG/Rij rat is presented in Table 4. Here, what is shown are the pharmacological effects of agonist or antagonist of group I/II/III in terms of SWDs incidence in both animal models. Based on the outcomes of Table 4, it can be seen that the role of mGluR receptors is not uniform in two different absence epilepsy models. Indeed, agonists of group II are anti absence drugs in the lh/lh model, in WAG/Rij rat the same agonists are pro absence, while, group II/III antagonist are anti absence in the WAG/Rij model. In lh/lh mice, no group I or III agonist has been exposed to test, and until now no group I agonist or antagonist has been tested in the WAG/Rij model. Considering the location of mGluR in the C-T-C network and the effects in the mice model, it might be expected that group I PAMs/NAMs could exert an effect on SWDs. More specifically, a Group I agonist should be pro-absence since the antagonists hava an anti-absence action. Another way of reasoning and also based on the results as presented in Table 4 regarding the opposite action of group II drugs (as has been demonstrated for LY379268) in the two models and the opposite action of Group I and Group II agonists on absence epilepsy, a anti-absence action is predicted for Group I agonists in the WAG/Rij model.

**Table 3** [1] Moldrich et al., 2003; [2] Shannon et al., 2005; 1998; [3] Burgess et al., 1997; [4] Chapman et al., 1999; [5] Chapman et al., 2000; [6] Lojkova´ and Mares., 2005; [7] Nagaraja and Grecksch., 2004; [8] Borowicz et al., 2003.

| | CONVULSIVE EPILEPSY | CONVULSIVE EPILEPSY | NON-CONVULSIVE EPILEPSY | NON-CONVULSIVE EPILEPSY (Lh/Lh mice | LOW-DOSE PTZ | HIGH-DOSE PTZ |
|---|---|---|---|---|---|---|
| | **Orthosteric Agonist** | **Orthosteric Antagonist** | **Orthosteric Agonist** | **Orthosteric Antagonist** | **Orthosteric Agonist** | **Orthosteric Agonist** |
| mGlu1 | [1]DHPG<br>[1]Quisqualate | [1]AIDA<br>[1]LY367385<br>[2]LY456236 | n.a | [3]AIDA<br>[3]LY367385 | [4]AIDA<br>[4]LY367385 | [7]AIDA 7LY367385<br>(no effect) |
| mGlu5 | [1]CHPG<br>[1]Quisqualate | [4]SIB1893 | n.a | [5]MPEP<br>[6](also NMDA antagonist and mGlu4R PAM) MTEP | [5]MPEP | [8]SIB 1893<br>(no effect) |

**Table 4** [1]Burgess et al., 1997; [2]Chapman et al., 1999; [3]Chapman et al., 2000; [4]Lojkova´ and Mares., 2005; [5]Cheong et al., 2009; [6]Ngomba et al., 2005; [7]Moldrich et al., 2001; [8]Ngomba et al., 2008; [9]Ngomba et al., 2011.

| | Ih/Ih mice | | WAG/Rij rat | |
|---|---|---|---|---|
| | **Agonist** | **Antagonist** | **Agonist** | **Antagonist** |
| Group I | n.a | [1] AIDA<br>[2]LY367385<br>[3-4] MPEP (mGlu5 NAM/ weak mGlu4 PAM)<br>[5] Deletion of PLCβ4 in the thalamocortical relay nuclei leads to absence seizure ↓ | n.a | n.a |
| Group II | [7]LY379268 ↓ | n.a | [8] LY379268 ↑ | [6] LY341495 ↓ |
| Group III | n.a | n.a | [9] PHCCC ↑ | n.a |

### Possible clinical use of metabotropic allosteric modulators

So far, no GPCR allosteric modulators have been authorized for the treatment of psychiatric and neurological disorders. On the other hand, multiple allosteric modulators have been submitted for clinical development. mGlu5 receptor PAMs are already being developed for use in the treatment of schizophrenia, with the only concern being the neurotoxicity and convulsive seizures provoked by very high doses of these compounds (Parmentier-Batteur et al., 2014). Phase II clinical trials have recently revealed promising effects of mGluR5 NAMs when treating anxiety and affective disorders, Parkinsons's disease and fragile X syndrome (Emmitte, K.A. a patent review 2010-2012; Rocher et al., 2011).

## Aim and outline of the thesis

### The aim of this thesis:

The experiments, as described in this thesis investigate the involvement of group I mGluR (mGluR1 and mGluR5 respectively) in the pathophysiology of absence epilepsy. This will be done in a well-established and often used model: rats of the WAG/Rij strain. The role of this class of receptors will be investigated by establishing their expression and signaling in the brain regions where the pathological SWDs initiate, spread and are maintained: the

C-T-C network. Furthermore, a series of pharmacological EEG-behavioural studies will be carried out with the aim to establish whether PAMs or NAMs of this group can be developed as putative anti-absence drugs. These compounds might be useful as drugs for the treatment of absence epilepsy, in particular for those patients who are refractory to conventional treatment. In tangible terms, the following steps are taken to realize the above goals:

In **Chapter 2** the role of type-1 mGluR in the occurrence on SWDs will be evaluated. First the expression and signaling of mGlu1 receptors will be evaluated in thalamic nuclei. Immunohistochemical techniques will be used for a precise localization of the mGlu1 receptor expression seen in symptomatic rats in the dorsal, medial and lateral complex of the thalamus of WAG/Rij and control rats. An *in vivo* method (*in situ* hybridization), the DHPG-stimulated PI hydrolysis, will be used in order to evaluate whether mGlu1 receptor signaling is odd in the thalamus of WAG/Rij rats. Next, the effects of the selective receptor mGlu1 enhancer SYN119 at the doses of 3, 10 and 30 mg/kg, s.c., and with an antagonist, JNJ16259685 (2.5 and 5mg/kg, i.p.) on the SWDs occurrence in symptomatic 8-month-old rats will be examined. Finally, in order to see if motor side effects occur after drug administration, the behavior of the rats will be quantified before, as well as after drug administration.

In **Chapter 3** the role of mGlu5 receptors in absence epilepsy will be elucidated. The study will be extended to mGluR5, by using a novel enhancer, the VU0360172 PAM, which may adjust the abnormalities in mGlu5 receptor signaling without recruiting "silent" mGlu5 receptors that might arise dose-related adverse effects as the orthosteric compounds does. It should be emphasized that more than 20% of patients with absence epilepsy syndrome and those with atypical absence seizure are refractory to conventional medications (reviewed Niswender and Conn, 2010).

From a therapeutic stand point of view, it is crucial to establish whether putative anti-epileptic drugs will develop tolerance since medication will be given chronically. Establishing tolerance is important for the preclinical and clinical development of mGlu receptor PAMs for the treatment of absence epilepsy in humans.

In **Chapter 4** tolerance will be investigated. VU0360172 (mGlu5 PAM) or RO0711401 (mGlu1 PAM) will be given to rats twice a day for ten days, followed by a 2 day wash out period, then a challenge with each of the two drugs will be performed after 3 days of withdrawal. EEG and behavior will be recorded during all days of the study.

In order to explain the receptor selectivity in the adaptive changes caused by the two PAMs, pharmacodynamic effects of the two compounds will be established in a parallel study, in which the expression of mGlu1α and mGlu5 receptors in the thalamus and cortex will be measured. This analysis will also be carried out on non epileptic Wistar rats treated with the two drugs. Next, the concentration of RO0711401 and VU0360172 in cortex and thalamus via Liquid Chromatography–tandem mass spectrometry analysis will be evaluated.

In **Chapter 5** a C-T-C circuit demarcation strategy will be adopted by independent bilateral intra-cortex or intra-thalamus micro-infusions to investigate site-specific effects on the regulation of SWDs in symptomatic WAG/Rij rats. In order to do so, a micro-infusion of RO0711401 or VU030172 PAMs will be performed. Moreover, it is well known that not only the glutamatergic neurotransmission is known to be one of the causes responsible for the initiation and spread of seizures, but also the GABAergic neurotransmission and often glutamate and GABA interact at the same synapses. Therefore tiagabine, a GABA reuptake inhibitor at the neuronal or glial GAT-1 transporter, will be micro-injected and in combination with VU0360172 to examine whether GABA alone, or in combination with VU0360172, and in which direction, an enhanced availability of extra-synaptic GABA influences responses to VU0360172, in thalamus and somatosensory cortex.

In **Chapter 6** "Is there a future for mGlu5 PAMs in absence epilepsy? a comparison with Ethosuximide", is made. Here the need for new treatment options in absence epilepsy is emphasized since 47% of subjects treated with ETX failed in therapy. The assumed working mechanisms of ETX, the neurochemical and pharmacological studies of the new anti-absence drugs mGlu1 and mGlu5 PAMs, RO0711401 and VU0360172, are described and also a comparison between ETX and VU0360172 is fully given.

**Chapter 7:** it is often difficult good to get good seizure control with only a single antiepileptic drug and therefore interaction studies between different antiepileptic drugs are indicated. Moreover, antiepileptic drugs may alter cognition and psychomotor functioning. A primary goal of epileptology is to recognize treatments that are not just symptomatic, but disease-modifying. It is well known that ESM has become the drug of choice in the treatment of patients with absence seizures taking into account its efficacy, tolerability and antiepileptogenic properties in man and in genetic absence models. Here it will be investigated if anti-epileptogenesis, induced by chronic ESM interacts with the putative anti-absence drug VU0360172. Next, a biochemistry analysis of the receptors expression will be done in cortex and thalamus in young presymptomatic WAG/Rij rats and in the chronically ESM treated rats. Finally, a learning task was performed to investigate the impact of antiepileptogenesis on cognition.

# References


Aizawa M, Ito Y, Fukuda H. 1997. Pharmacological profiles of generalized absence seizures in lethargic, stargazer and gamma-hydroxybutyrate-treated model mice. Neurosci Res. 29, 17-25.

Alexander GM, Godwin DW., 2006. Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res. 71, 1-22.

Ayala, J. E. et al. 2009. mGluR5 positive allosteric modulators facilitate both hippocampal LTP and LTD and enhance spatial learning. Neuropsychopharmacology 34, 2057-71.

Bambal, G. Cakil, D. Ekici, F., 2011. Models of experimental epilepsy. J Clin Exp Invest 2, 118- 23.

Banerjee. 2009. The descriptive epidemiology of epilepsy-a review. Epilepsy Res. 85, 31-45.

Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P. 1993. The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 4, 771-87.

Bear, M.F., Huber, K.M. & Warren, S,T. 2004. The mGluR theory of fragile X mental retardation. Trends Neurosci 27, 370-7.

Benbadis SR, Agrawal V, Tatum WO 4th. 2001. How many patients with psychogenic non epileptic seizures also have epilepsy? Neurology 5, 915-7.

Berg AT, Levy SR, Testa FM, Blumenfeld H. 2014. Long-term seizure remission in childhood absence epilepsy: might initial treatment matter? Epilepsia 55, 551-7.

Berg AT, Millichap JJ. 2013. The 2010 revised classification of seizures and epilepsy. Continuum (Minneap Minn) 19, 571-97.

Berg AT, Berkovic SF, Brodie MJ, Buchhalter J, Cross JH, van Emde Boas W, Engel J, French J, Glauser TA, Mathern GW, Moshé SL, Nordli D, Plouin P, Scheffer IE. 2010. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE Commission on Classification and Terminology, 2005-2009. Epilepsia. 51, 676-85.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F. 2010. Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 3, 18-53.

Bertaso F, Zhang C, Scheschonka A, de Bock F, Fontanaud P, Marin P, Huganir RL, Betz H, Bockaert J, Fagni L, Lerner-Natoli M. 2008. PICK1 uncoupling from mGluR7a causes absence-like seizures. Nat Neurosci 8, 940-8.

Blumenfeld H, Klein JP, Schridde U, Vestal M, Rice T, Khera DS, Bashyal C,Giblin K, Paul-Laughinghouse C, Wang F, Phadke A, Mission J, Agarwal RK, Englot DJ, Motelow J, Nersesyan H, Waxman SG, Levin AR. 2008. Early treatment suppresses the development of spike-wave epilepsy in a rat model. Epilepsia 49, 400-9.

Blumenfeld H. 2005. Cellular and network mechanisms of spike-wave seizures Epilepsia 46, 21-33.

Borowicz KK, Piskorska B, Łuszczki J, Czuczwar SJ. 2003. Influence of SIB 1893, a selective mGluR5 receptor antagonist, on the anticonvulsant activity of conventional antiepileptic drugs in two models of experimental epilepsy. Pol J Pharmacol. 55, 735-40.

Bosnyakova D, Gabova A, Zharikova A, Gnezditski V, Kuznetsova G, van Luijtelaar G. 2007. Some peculiarities of time-frequency dynamics of spike-wave discharges in humans and rats. Clin Neurophysiol. 118, 1736-43

Bruno V, Battaglia G, Copani A, D'Onofrio M, Di Iorio P, De Blasi A, Melchiorri D, Flor PJ, Nicoletti F. 2001. Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J Cereb Blood 9, 1013-33.

Burgess DL, Noebels JL. 1999. Single gene defects in mice: the role of voltage-dependent calcium channels in absence models. Epilepsy Res. 36, 111-22.

Buzsâki G, Laszlovszky I, Lajtha A, Vadâsz C .1990a. Spike-and-wave neocortical patterns in rats: genetic and aminergic control. Neuroscience 38, 323–333.

Buzsâki G, Smith A, Berger S, Fisher LJ, Gage FH. 1990b. Petit mal epilepsy and parkinsonian tremor: hypothesis of a common pacemaker. Neuroscience 36, 1–14.

Byun NE, Grannan M, Bubser M, Barry RL, Thompson A, Rosanelli J, Gowrishankar R, Kelm ND, Damon S, Bridges TM, Melancon BJ, Tarr JC, Brogan JT, Avison MJ, Deutch AY, Wess J, Wood MR, Lindsley CW, Gore JC, Conn PJ, Jones CK. 2014. Antipsychotic drug-like effects of the selective M4 muscarinic acetylcholine receptor positive allosteric modulator VU0152100. Neuropsychopharmacol. 39, 1578-93.

Campbell DB, North JB, Hess EJ. 1999. Tottering mouse motor dysfunction is abolished on the Purkinje cell degeneration (pcd) mutant background. Exp Neurol. 160, 268-78

Carmant L, Kramer U, Holmes GL, Mikati MA, Riviello JJ, Helmers SL. 1996.Differential diagnosis of staring spells in children: a video-EEG study. Pediatr Neurol. 14, 199-202.

Caulder EH, Riegle MA, Godwin DW. 2013. Activation of group 2 metabotropic glutamate receptors reduces behavioral and electrographic correlates of pilocarpine induced status epilepticus. Epilepsy Res. 108, 171-81.

Cavalheiro EA, Santos NF, Priel MR. 1996. The pilocarpine model of epilepsy in mice. Epilepsia 37, 1015-9.

Chadwick D, Smith D. 2002 The misdiagnosis of epilepsy. Brithis Medical Journal 324, 495-6.

Chahboune H, Mishra AM, DeSalvo MN, Staib LH, Purcaro M, Scheinost D,Papademetris X, Fyson SJ, Lorincz ML, Crunelli V, Hyder F, Blumenfeld H. 2009. DTI abnormalities in anterior corpus callosum of rats with spike-wave epilepsy. Neuroimage 47, 459-66.

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJ, Bymaster FP, Felder CC. 2008. Allosteric modulation of the muscarinic $M_4$ receptor as an approach to treating schizophrenia. Proc. Natl Acad. Sci. USA 105, 10978-83.

Chapman AG, Dürmüller N, Harrison BL, Baron BM, Parvez N, Meldrum BS. 1995. Anticonvulsant activity of a novel NMDA/glycine site antagonist, MDL 104,653, against kindled and sound-induced seizures. Eur J Pharmacol. 274, 83-8.

Chapman AG, Nanan K, Williams M, Meldrum BS. 2000. Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacology 39, 1567-74.

Chapman AG, Yip PK, Yap JS, Quinn LP, Tang E, Harris JR, Meldrum BS. 1999. Anticonvulsant actions of LY 367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid). Eur J Pharmacol. 368, 17-24.

Charpier S, Leresche N, Deniau JM, Mahon S, Hughes SW, Crunelli V. 1999. On the putative contribution of GABA(B) receptors to the electrical events occurring during spontaneous spike and wave discharges. Neuropharmacology 38, 1699-706.

Chen J, Larionov S, Pitsch J, Hoerold N, Ullmann C, Elger CE, Schramm J, Becker AJ. 2005. Expression analysis of metabotropic glutamate receptors I and III in mouse strains with different susceptibility to experimental temporal lobe epilepsy. Neurosci Lett. 375, 192-7.

Chocholová L. 1983. Incidence and development of rhythmic episodic activity in the electroencephalogram of a large rat population under chronic conditions. Physiol Bohemoslov. 32, 10-8.

Coenen AM, van Luijtelaar EL. 1987.The WAG/Rij rat model for absence epilepsy: age and sex factors. Epilepsy Res. 1, 297-301.

Coenen AM, van Luijtelaar EL. 2003. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav Genet. 33, 635-55.

Collins TF. 1972. Effect of captan and triethylenemelamine (TEM) on reproductive fitness of DBA-2J mice. Toxicol Appl Pharmacol. 23, 277-87.

Conn PJ, Pin JP. 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol. 37, 205-37.

Costa MS, Rocha JB, Perosa SR, Cavalheiro EA, Naffah-Mazzacoratti Mda G. 2004.Pilocarpine-induced status epilepticus increases glutamate release in rat hippocampal synaptosomes. Neurosci Lett. 356, 41-4.

Crunelli V, Leresche N. 2002. Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci. 3, 371-82.

Curia G, Longo D, Biagini G, Jones RS, Avoli M. 2008. The pilocarpine model of temporal lobe epilepsy. J Neurosci Methods. 172, 143-57.

Dalby NO, Thomsen C. 1996. Modulation of seizure activity in mice by metabotropic glutamate receptor ligands. J Pharmacol Exp Ther. 276, 516-22.

De Sarro G, Nava F, Aguglia U, De Sarro A. 1996. Lamotrigine potentiates the antiseizure activity of some anticonvulsants in DBA/2 mice. Neuropharmacology 35, 153-8.

De Sarro G, Russo E, Citraro R, Meldrum BS. 2015. Genetically epilepsy-prone rats (GEPRs) and DBA/2 mice: Two animal models of audiogenic reflex epilepsy for the evaluation of new generation AEDs. Epilepsy Behav. doi:10.1016/j.yebeh.2015.06.030

Depaulis, A, van Luijtelaar, G. Genetic models of absence epilepsy, in: Pitkanen, A., Schwartzkroin, P.A., Mosche, S.L. (Ed.). 2006. Models of Seizure and Epilepsy Elsevier Academic Press, San Diego. pp 233–248

De Sarro G, Siniscalchi A, Ferreri G, Gallelli L, De Sarro A. 2010. NMDA and AMPA/kainate receptors are involved in the anticonvulsant activity of riluzole in DBA/2 mice. Eur J Pharmacol. 408, 25-34.

Emmitte KA. 2013. mGlu5 negative allosteric modulators: a patent review (2010-2012). Expert Opin Ther Pat. 23, 393-408.

Estrada FS, Hernández VS, López-Hernández E, Corona-Morales AA, Solís H, Escobar A, Zhang L. 2012. Glial activation in a pilocarpine rat model for epileptogenesis: a morphometric and quantitative analysis. Neurosci Lett. 514, 51-6.

Ferraguti F, Crepaldi L, Nicoletti F. 2008. Metabotropic glutamate 1 receptor: current concepts and perspectives. Pharmacol Rev. 60, 536-81.

Ferraguti F, Shigemoto R. 2006. Metabotropic glutamate receptors. Cell Tissue Res. 326, 483-504.

Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, Engel J Jr, Forsgren L, French JA, Glynn M, Hesdorffer DC, Lee BI, Mathern GW, Moshé SL, Perucca E, Scheffer IE, Tomson T, Watanabe M, Wiebe S. 2014. ILAE official report: a practical clinical definition of epilepsy. Epilepsia 55, 475-82.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy JD Jr, Hawkes R, Frankel WN, Copeland NG, Jenkins NA. 1996. Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 87, 607-17.

Forsgren L. 1992. Prevalence of epilepsy in adults in northern Sweden. Epilepsia 33, 450-8.

Gao B, Sekido Y, Maximov A, Saad M, Forgacs E, Latif F, Wei MH, Lerman M, Lee JH, Perez-Reyes E, Bezprozvanny I, Minna JD. 2000. Functional properties of a new voltage-dependent calcium channel alpha(2)delta auxiliary subunit gene (CACNA2D2). J Biol Chem. 275, 12237-42.

Gasparini F, Lingenhöhl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I, Biollaz M, Allgeier H, Heckendorn R, Urwyler S, Varney MA, Johnson EC, Hess SD, Rao SP, Sacaan AI, Santori EM, Veliçelebi G, Kuhn R. 1999. 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology 38, 1493-503.

Gastambide F, Gilmour G, Robbins TW, Tricklebank MD.2013.The mGlu5 positive allosteric modulator LSN2463359 differentially modulates motor, instrumental and cognitive effects of NMDA receptor antagonists in the rat. Neuropharmacology 64, 240-7.

Gastaut H. 1969. Classification of the epilepsies. Proposal for an international classification. Epilepsia 10, 14-21.

Glauser TA, Cnaan A, Shinnar S, Hirtz DG, Dlugos D, Masur D, Clark PO,Capparelli EV, Adamson PC. 2010. Childhood Absence Epilepsy Study Group. Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. N Engl J Med. 362,790-9.

Gloor P. 1986. Consciousness as a neurological concept in epileptology: a critical review. Epilepsia 36, 499-515.

Godwin DW, Van Horn SC, Eriir A, Sesma M, Romano C, Sherman SM. 1996. Ultrastructural localization suggests that retinal and cortical inputs access different metabotropic glutamate receptors in the lateral geniculate nucleus. J Neurosci. 16, 8181-92.

Hermans E, Challiss RA. 2001. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. Biochem J. 359, 465-84.

Hermans E, Challiss RA. 2001. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. Biochem J. 359, 465-84.

Hesdorffer D, Beghi E. 2011. ILAE epidemiology commission report: introduction to the supplement. Epilepsia 52 Suppl 7:1.

Hesdorffer DC, Tomson T, Benn E, Sander JW, Nilsson L, Langan Y, Walczak TS, Beghi E, Brodie MJ, Hauser WA; ILAE Commission on Epidemiology (Subcommission on Mortality). 2012. Do antiepileptic drugs or generalized tonic-clonic seizure frequency increase SUDEP risk? A combined analysis. Epilepsia 53, 249-52.

Hildebrand ME, David LS, Hamid J, Mulatz K, Garcia E, Zamponi GW, Snutch TP. 2007. Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11-coupled muscarinic acetylcholine receptors. J Biol Chem. 282, 21043-55.

Hildebrand ME, Isope P, Miyazaki T, Nakaya T, Garcia E, Feltz A, Schneider T, Hescheler J, Kano M, Sakimura K, Watanabe M, Dieudonné S, Snutch TP. 2009. Functional coupling between mGluR1 and Cav3.1 T-type calcium channels contributes to parallel fiber-induced fast calcium signaling within Purkinje cell dendritic spines. J Neurosci. 29, 9668-82.

Hosford DA, Clark S, Cao Z, Wilson WA Jr, Lin FH, Morrisett RA, Huin A. 1992. The role of GABAB receptor activation in absence seizures of lethargic (lh/lh) mice. Science 257, 398-401.

Hosford DA, Wang Y. 1997. Utility of the lethargic (lh/lh) mouse model of absence seizures in predicting the effects of lamotrigine, vigabatrin, tiagabine, gabapentin, and topiramate against human absence seizures. Epilepsia 38, 408-14.

Huber, K.M.,Roder, J.C.and Bear, M.F. 2001. Chemical induction long-term depression in hippocampal area CA1. J.Neurophysiol. 86, 321-325.

Hughes SW, Cope DW, Blethyn KL, Crunelli V. 2002. Cellular mechanisms of the slow (<1 Hz) oscillation in thalamocortical neurons in vitro. Neuron 33, 947-58.

Huguenard JR. 2002. Block of T -Type Ca(2+) Channels Is an Important Action of Succinimide Antiabsence Drugs. Epilepsy Curr. 2, 49-52.

Inoue M, Peeters BW, van Luijtelaar EL, Vossen JM, Coenen AM. 1990. Spontaneous occurrence of spike-wave discharges in five inbred strains of rats. Physiol Behav. 48, 199-201.

Izzi C, Barbon A, Toliat MR, Heils A, Becker C, Nürnberg P, Sander T, Barlati S. 2003. Candidate gene analysis of the human metabotropic glutamate receptor type 4 (GRM4) in patients with juvenile myoclonicepilepsy. Am J Med Genet B Neuropsychiatr Genet. 123, 59-63.

Johnson BG, Wright RA, Arnold MB, Wheeler WJ, Ornstein PL, Schoepp DD. 1999. [3H]-LY341495 as a novel antagonist radioligand for group II metabotropic glutamate (mGlu) receptors: characterization of binding to membranes of mGlu receptor subtype expressing cells. Neuropharmacology 38, 1519-29.

Kaminski RM, Rogawski MA, Klitgaard H. 2014. The potential of antiseizure drugs and agents that act on novel molecular targets as antiepileptogenic treatments. Neurotherapeutics 11, 385-400.

Klingberg F, Pickenhain L.1968. The appearance of "spindle-activity" in rat in correlation to behaviour. Act Nerv Super (Praha) 10, 203-4.

Linden AM, Johnson BG, Peters SC, Shannon HE, Tian M, Wang Y, Yu JL, Köster A, Baez M, Schoepp DD. 2002. Increased anxiety-related behavior in mice deficient for metabotropic glutamate 8 (mGlu8) receptor. Neuropharmacology 43, 251-9.

Litschig S, Gasparini F, Rueegg D, Stoehr N, Flor PJ, Vranesic I, Prézeau L, Pin JP, Thomsen C, Kuhn R. 1999. CPCCOEt, a non competitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. Mol. Pharmacol. 55, 453-61.

Liu XB, MuÇoz A, Jones EG. 1998. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol. 395, 450-65.

Loiseau P. 1992. Human absence epilepsies. J Neural Transm Suppl. 35, 1-6.

Loiseau P, Panayiotopolus CP, Hirsch E. 2002. Childhood absence epilepsy and related syndromes. In: Roger J, Bureau M, Dravet C et al, eds. Epileptic Syndromes in Infancy, Childhood and Adolescence (3rd edn).3rd ed. London: John Libbey & Co Ltd 285-304.

Löscher W, Brandt C. 2009. High seizure frequency prior to antiepileptic treatment is a predictor of pharmacoresistant epilepsy in a rat model of temporal lobe epilepsy. Epilepsia 51, 89-97.

Löscher W, Hoffmann K, Twele F, Potschka H, Töllner K. 2013. The novel antiepileptic drug imepitoin compares favourably to other GABA-mimetic drugs in a seizure threshold model in mice and dogs. Pharmacol Res 77, 39-46.

Löscher W, Schmidt D. 2006. Experimental and clinical evidence for loss of effect (tolerance) during prolonged treatment with antiepileptic drugs. Epilepsia 47, 1253-84.

Löscher W. 1982 Relationship between GABA concentrations in cerebrospinal fluid and seizure excitability. J Neurochem. 38, 293-5.

Lüders H. 2004. Brain stimulation and epilepsy: novel approaches for seizure control. Suppl Clin Neurophysiol. 57, 379-82.

Lüders HO. Tufenkjian K. 2012. Seizure semiology: its value and limitations in localizing the epileptogenic zone. J Clin Neurol. 8, 243-50.

Lüttjohann A, Fabene PF, van Luijtelaar G. 2009. A revised Racine's scale for PTZ-induced seizures in rats. Physiol Behav. 98, 579-86.

Manning JP, Richards DA, Bowery NG. 2003. Pharmacology of absence epilepsy. Trends Pharmacol Sci. 10, 542-9.

Marescaux C, Vergnes M, Bernasconi R. 1992. GABAB receptor antagonists: potential new anti-absence drugs. J Neural Transm Suppl. 35, 179-88.

Marescaux C, Vergnes M, Depaulis A. 1992. Genetic absence epilepsy in rats from Strasbourg -a review. J Neural Transm Suppl. 35, 37-69.

Martin LJ, Blackstone CD, Huganir RL, Price DL. 1992. Cellular localization of a metabotropic glutamate receptor in rat brain. Neuron. 2, 259-70.

McCormick DA, Bal T. 1997. Sleep and arousal: thalamocortical mechanisms. Annu Rev Neurosci. 20, 185-215.

Meeren H, van Luijtelaar G, Lopes da Silva F, Coenen A. 2005. Evolving concepts on the pathophysiology of absence seizures: the cortical focus theory. Arch Neurol. 62, 371-6.

Meeren HK, Pijn JP, Van Luijtelaar EL, Coenen AM, Lopes da Silva FH. 2002. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci. 22, 1480-95.

Meldrum BS, Chapman AG. 1999. Excitatory amino acid receptors and antiepileptic drug development. Adv Neurol. 79, 965-78.

Moldrich RX, Chapman AG, De Sarro G, Meldrum BS. 2003. Glutamate metabotropic receptors as targets for drug therapy in epilepsy. Eur J Pharmacol. 476, 3-16.

Moldrich RX, Talebi A, Beart PM, Chapman AG, Meldrum BS. 2001.The mGlu(2/3) agonist 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate, is anti- and proconvulsant in DBA/2 mice. Neurosci Lett. 299, 1-2.

Morgans CW, Brown RL, Duvoisin RM. 2010. TRPM1: the endpoint of the mGluR6 signal transduction cascade in retinal ON-bipolar cells. Bioessays 32, 609–14.

Movsesyan VA, O'Leary DM, Fan L, Bao W, Mullins PG, Knoblach SM, Faden AI. 2001. mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine and (E)-2-methyl-6-(2-phenylethenyl)-pyridine reduce traumatic neuronal injury in vitro and in vivo by antagonizing N-methyl-D-aspartate receptors. J Pharmacol Exp Ther. 296, 41-7.

Mukherjee S, Manahan-Vaughan D. 2013. Role of metabotropic glutamate receptors in persistent forms of hippocampal plasticity and learning. Neuropharmacology. 66, 65-81.

Nagaraja RY, Grecksch G, Reymann KG, Schroeder H, Becker A. 2004. Group I metabotropic glutamate receptors interfere in different ways with pentylenetetrazole seizures, kindling, and kindling-related learning deficits. Naunyn Schmiedebergs Arch Pharmacol. 370 ,26-34.

Ngomba RT, Biagioni F, Casciato S, Willems-van Bree E, Battaglia G, Bruno V, Nicoletti F, van Luijtelaar EL. 2005.The preferential mGlu2/3 receptor antagonist,LY341495, reduces the frequency of spike-wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology. 49, 89-103.

Ngomba RT, Biagioni F, Casciato S, Willems-van Bree E, Battaglia G, Bruno V, Nicoletti F, van Luijtelaar EL. 2005. The preferential mGlu2/3 receptor antagonist, LY341495, reduces the frequency of spike-wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology. 49, 89-103.

Ngomba RT, Ferraguti F, Badura A, Citraro R, Santolini I, Battaglia G, Bruno V, De Sarro G, Simonyi A, van Luijtelaar G, Nicoletti F. 2008. Positive allosteric modulation of metabotropic glutamate 4 (mGlu4) receptors enhances spontaneous and evoked absence seizures. Neuropharmacology 54, 344-54.

Ngomba RT, Santolini I, Biagioni F, Molinaro G, Simonyi A, van Rijn CM, D'Amore V, Mastroiacovo F, Olivieri G, Gradini R, Ferraguti F, Battaglia G, Bruno V, Puliti A, van Luijtelaar G, Nicoletti F. 2011. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology 60, 1281-91.

Niswender CM, Conn PJ.2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol 50, 295-322.

Niswender CM, Johnson KA, Weaver CD, Jones CK, Xiang Z, Luo Q, Rodriguez AL,Marlo JE, de Paulis T, Thompson AD, Days EL, Nalywajko T, Austin CA, Williams MB, Ayala JE, Williams R, Lindsley CW, Conn PJ. 2008. Discovery, characterization, and antipakinsonian effect of novel positive allosteric modulators of metabotropic glutamate receptor 4. Mol. Pharmacol. 74, 1345-58.

Noetzel MJ, Rook JM, Vinson PN, Cho HP, Days E, Zhou Y, Rodriguez AL, Lavreysen H, Stauffer SR, Niswender CM, Xiang Z, Daniels JS, Jones CK, Lindsley CW, Weaver CD, Conn PJ. 2012. Functional impact of allosteric agonist activity of selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 in regulating central nervous system function. Mol. Pharmacol. 81, 120-33.

Nolan M, Bergazar M, Chu B, Cortez MA, Snead OC 3rd. 2005. Clinical and neurophysiologic spectrum associated with atypical absence seizures in children with intractable epilepsy. J Child Neurol. 20, 404-10.

O'Brien JA, Lemaire W, Chen TB, Chang RS, Jacobson MA, Ha SN, Lindsley CW, Schaffhauser HJ, Sur C, Pettibone DJ, Conn PJ, Williams DL Jr. 2003. A family of highly selective allosteric modulators of the metabotropic glutamate receptor subtype 5. Mol. Pharmacol. 64, 731-740.

O'Leary DM, Movsesyan V, Vicini S, Faden AI. 2000. Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism. Br J Pharmacol. 131, 1429-37.

Onat FY, van Luijtelaar G, Nehlig A, Snead OC 3rd. 2013. The involvement of limbic structures in typical and atypical absence epilepsy. Epilepsy Res. 103, 111-23.

Osten P, Stern-Bach Y. 2006. Learning from stargazin: the mouse, the phenotype and the unexpected. Curr Opin Neurobiol. 3, 275-80.

Panayiotopoulos CP. 1999. Typical absence seizures and their treatment. Arch Dis Child. 81, 351-5.

Panayiotopoulos CP. 2001. Treatment of typical absence seizures and related epileptic syndromes. Paediatr Drugs. 3, 379-403.

Parmentier-Batteur S, Hutson PH, Menzel K, Uslaner JM, Mattson BA, O'Brien JA,Magliaro BC, Forest T, Stump CA, Tynebor RM, Anthony NJ, Tucker TJ, Zhang XF,Gomez R, Huszar SL, Lambeng N, Fauré H, Le Poul E, Poli S, Rosahl TW, Rocher JP, Hargreaves R, Williams TM. 2014. Mechanism based neurotoxicity of mGlu5 positive allosteric modulators--development challenges for a promising novel antipsychotic target. Neuropharmacology. 82, 161-73.

Paz JT, Bryant AS, Peng K, Fenno L, Yizhar O, Frankel WN, Deisseroth K, Huguenard JR. 2011. A new mode of corticothalamic transmission revealed in the Gria4(-/-) model of absence epilepsy. Nat Neurosci. 14, 1167-73.

Peeters BW, Kerbusch JM, Coenen AM, Vossen JM, van Luijtelaar EL. 1992. Genetics of spike-wave discharges in the electroencephalogram (EEG) of the WAG/Rij inbred rat strain: a classical mendelian crossbreeding study. Behav Genet. 22, 361-8.

Peeters BW, Ramakers GM, Ellenbroek BA, Vossen JM, Coenen AM. 1994a. Interactions between NMDA and nonNMDA receptors in nonconvulsive epilepsy in the WAG/Rij inbred strain. Brain Res Bull. 33, 715-8.

Peeters BW, Ramakers GM, Vossen JM, Coenen AM. 1994b.The WAG/Rij rat model for nonconvulsive absence epilepsy: involvement of nonNMDA receptors. Brain Res Bull. 33, 709-13.

Perez-Mendes P, Blanco MM, Calcagnotto ME, Cinini SM, Bachiega J, Papoti D, Covolan L, Tannus A, Mello LE. 2011. Modeling epileptogenesis and temporal lobe epilepsy in a non-human primate. Epilepsy Res. 96, 45-57.

Pin JP, Duvoisin R. 1995.The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34, 1–26.

Pin JP, Kniazeff J, Liu J, Binet V, Goudet C, Rondard P, Pr_zeau L. 2005. Allosteric functioning of dimeric class C G-protein-coupled receptors. FEBS J 272, 2947-55.

Pitkänen A, Bolkvadze T. Head Trauma and Epilepsy. In: Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, editors. Jasper's. 2012. Basic Mechanisms of the Epilepsies. 4th edition. Oxford University Press, Oxford, pp 331-342.

Pitkänen A, Philip.A. Schwartzkroin and Solomon L. Moshé. 2006. Models of seizures and epilepsy Elsevier Academic Press, London.

Pitsch J, Schoch S, Gueler N, Flor PJ, van der Putten H, Becker AJ. 2007. Functional role of mGluR1 and mGluR4 in pilocarpine-induced temporal lobe epilepsy. Neurobiol Dis. 26, 623-33.

Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S. 2007. Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. J Neurosci. 27, 6590-9.

Reichova I, Sherman SM. 2004. Somatosensory corticothalamic projections: distinguishing drivers from modulators. J Neurophysiol. 92, 2185-97.

Rivadulla C, Martínez LM, Varela C, Cudeiro J. 2002. Completing the corticofugal loop: a visual role for the corticogeniculate type 1 metabotropic glutamate receptor. J Neurosci 22, 2956-62.

Rocher JP, Bonnet B, Boléa C, Lütjens R, Le Poul E, Poli S, Epping-Jordan M, Bessis AS, Ludwig B, Mutel V. 2011. mGluR5 negative allosteric modulators overview: a medicinal chemistry approach towards a series of novel therapeutic agents. Curr Top Med Chem. 11, 680-95.

Rogawski MA, Löscher W. 2004. The neurobiology of antiepileptic drugs. Nat Rev Neurosci. 5, 553-64.

Rogawski MA. 1992. The NMDA receptor, NMDA antagonists and epilepsy therapy. A status report. Drugs. 44, 279-92.

Rudolf G, Bihoreau MT, Godfrey RF, Wilder SP, Cox RD, Lathrop M, Marescaux C, Gauguier D. 2004. Polygenic control of idiopathic generalized epilepsy phenotypes in the genetic absence rats from Strasbourg (GAERS). Epilepsia 45, 301-8.

Russo E, Citraro R, Scicchitano F, De Fazio S, Perrotta I, Di Paola ED, Constanti A, De Sarro G. 2011. Effects of early long-term treatment with antiepileptic drugs on development of seizures and depressive-like behavior in a rat genetic absence epilepsy model. Epilepsia 52,1341-50.

Salt TE, Binns KE. 2000.Contributions of mGlu1 and mGlu5 receptors to interactions with N-methyl-D-aspartate receptor-mediated responses and nociceptive sensory responses of rat thalamic neurones. Neuroscience 100, 375-80.

Salt TE, Eaton SA. 1996. Functions of ionotropic and metabotropic glutamate receptors in sensory transmission in the mammalian thalamus. Prog Neurobiol 48, 55-72.

Salt TE, Turner JP. 1998a. Reduction of sensory and metabotropic glutamate receptor responses in the thalamus by the novel mGluR1selective antagonist (S) 2-methyl-4-carboxy-phenylglycine. Neuroscience 85, 655-8.

Sander JW, Shorvon SD. 1996. Epidemiology of the epilepsies. 61, 433-43.

Sarkisian MR. 2001. Overview of the Current Animal Models for Human Seizure and Epileptic Disorders. Epilepsy Behav. 2, 201-216.

Schridde U, van Luijtelaar G. 2004. The influence of strain and housing on two types of spike-wave discharges in rats. Genes Brain Behav. 3, 1-7.

Schridde U, van Luijtelaar G. 2005 The role of the environment on the development of spike-wave discharges in two strains of rats. Physiol Behav. 84, 379-86.

Shannon HE, Peters SC, Kingston AE. 2005. Anticonvulsant effects of LY456236, a selective mGlu1 receptor antagonist. Neuropharmacology. 1, 188-95.

Shaw FZ. 2004. Is spontaneous high-voltage rhythmic spike discharge in Long Evans rats an absence-like seizure activity? J Neurophysiol. 91, 63-77.

Sherman SM, Guillery RW. 2002.The role of the thalamus in the flow of information to the cortex. Philos Trans R Soc Lond B Biol Sci. 357, 1695-708.

Shigemoto R, Nakanishi S, Mizuno N. 1992. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J Comp Neurol. 335, 252-66.

Sitnikova E, van Luijtelaar G. 2006. Cortical and thalamic coherence during spike-wave seizures in WAG/Rij rats. Epilepsy Res. 71, 159-80.

Sitnikova E, van Luijtelaar G. 2007. Electroencephalographic characterization of spike-wave discharges in cortex and thalamus in WAG/Rij rats. Epilepsia 12, 2296-31.

Snead OC 3rd. 1992. Evidence for G protein modulation of experimental-generalized absence seizures in rat. Neurosci Lett. 148, 15-8.

Snead OC III, Banerjee PK, Burnham M, Hampson D. 2000. Modulation of absence seizures by the GABA(A) receptor: a critical role for metabotropic glutamate receptor 4 (mGluR4). J Neurosci. 20, 6218-24.

Sperk G, Schwarzer C, Heilman J, Furtinger S, Reimer RJ, Edwards RH, Nelson N. 2013. Expression of plasma membrane GABA transporters but not of the vesicular GABA transporter in dentate granule cells after kainic acid seizures. Hippocampus 13, 806-15.

Tang FR, Lee WL, Gao H, Chen Y, Loh YT, Chia SC. 2004. Expression of different isoforms of protein kinase C in the rat hippocampus after pilocarpine-induced status epilepticus with special reference to CA1 area and the dentate gyrus. Hippocampus 14, 87-98.

Tenney JR, Jain SV. Absence Epilepsy: Older vs Newer AEDs. 2014. Curr Treat Options Neurol. 16, 290.

Thomsen C, Klitgaard H, Sheardown M, Jackson HC, Eskesen K, Jacobsen P, Treppendahl S, Suzdak PD. 1994. (S)-4-carboxy-3-hydroxyphenylglycine, an antagonist of metabotropic glutamate receptor (mGluR) 1a and an agonist of mGluR2, protects against audiogenic seizures in DBA/2 mice. J Neurochem. 62, 2492-5.

Urwyler, S. 2011. Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives. Pharmacol. Rev. 64, 59-126.

van Luijtelaar EL, Coenen AM. 1986. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett. 70, 393-7.

van Luijtelaar EL, de Bruijn SF, Declerck AC, Renier WO, Vossen JM, Coenen AM. 1991. Disturbances in time estimation during absence seizures in children. EpilepsyRes. 2, 148-53.

van Luijtelaar EL, Drinkenburg WH, van Rijn CM, Coenen AM. 2002. Rat models of genetic absence epilepsy: what do EEG spike-wave discharges tell us about drug effects? Methods Find Exp Clin Pharmacol. 24, 65-70.

van Luijtelaar G, Mishra AM, Edelbroek P, Coman D, Frankenmolen N, Schaapsmeerders P, Covolato G, Danielson N, Niermann H, Janeczko K, Kiemeneij A, Burinova J, Bashyal C, Coquillette M, Lüttjohann A, Hyder F, Blumenfeld H, van Rijn CM. 2013. Anti-epileptogenesis: Electrophysiology, diffusion tensor imaging and behavior in a genetic absence model. Neurobiol Dis. 60, 126-38.

van Luijtelaar G, Onat FY, Gallagher MJ. 2014. Animal models of absence epilepsies: what do they model and do sex and sex hormones matter? Neurobiol Dis.72, 167-79.

van Luijtelaar G, Sitnikova E. 2006. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 30, 983-1003.

van Luijtelaar, G., Sitnikova, k., Midzyanovskaya, I., Tolmacheva, E.A. 2007. Stress vulnerability and depressive symptoms in genetic absence epileptic rats, in: Hollaway, K.H. (Ed.), New Research on Epilepsy and Behaviour. Nova Science Pubblisher, Inc, New York 35, 854-76.

van Luijtelaar EL, Drinkenburg WH, van Rijn CM, Coenen AM. 2002. Rat models of genetic absence epilepsy: what do EEG spike-wave discharges tell us about drug effects? Methods Find Exp Clin Pharmacol. 24 Suppl D:65-70.

Varney MA, Cosford ND, Jachec C, Rao SP, Sacaan A, Lin FF, Bleicher L, Santori EM, Flor PJ, Allgeier H, Gasparini F, Kuhn R, Hess SD, Veliçelebi G, Johnson EC. 1999. SIB-1757 and SIB-1893: selective, noncompetitive antagonists of metabotropic glutamate receptor type 5. J Pharmacol Exp Ther. 290, 170-81.

Venit EL, Shepard BD, Seyfried TN. 2004. Oxygenation prevents sudden death in seizure-prone mice. Epilepsia 45, 993-6.

Vidnyanszky Z, Gorcs TJ, Negyessy L, Borostyankio Z, Knopfel T, Hamori J. 1996. Immunocytochemical visualization of the mGluR1a metabotropic glutamate receptor at synapses of corticothalamic terminals originating from area 17 of the rat. Eur J Neurosci. 6, 1061-71.

Vinogradova LV, van Rijn CM. 2015. Long-term disease-modifying effect of the endocannabinoid agonist WIN55, 212-2 in a rat model of audiogenic epilepsy. Pharmacol Rep. 67, 501-3.Vrielink ref is missing.

Vrielynck P. 2013. Current and emerging treatments for absence seizures in young patients. Neuropsychiatr Dis Treat. 9, 963-75.

Wang X, Ai J, Hampson DR, Snead OC III. 2005. Altered glutamate and GABA release within thalamocortical circuitry in metabotropic glutamate receptor 4 knockout mice. Neuroscience. 134, 1195-203.

Weiergräber M, Stephani U, Köhling R. 2010. Voltage-gated calcium channels in the etiopathogenesis and treatment of absence epilepsy. Brain Res Rev. 62, 245-71.

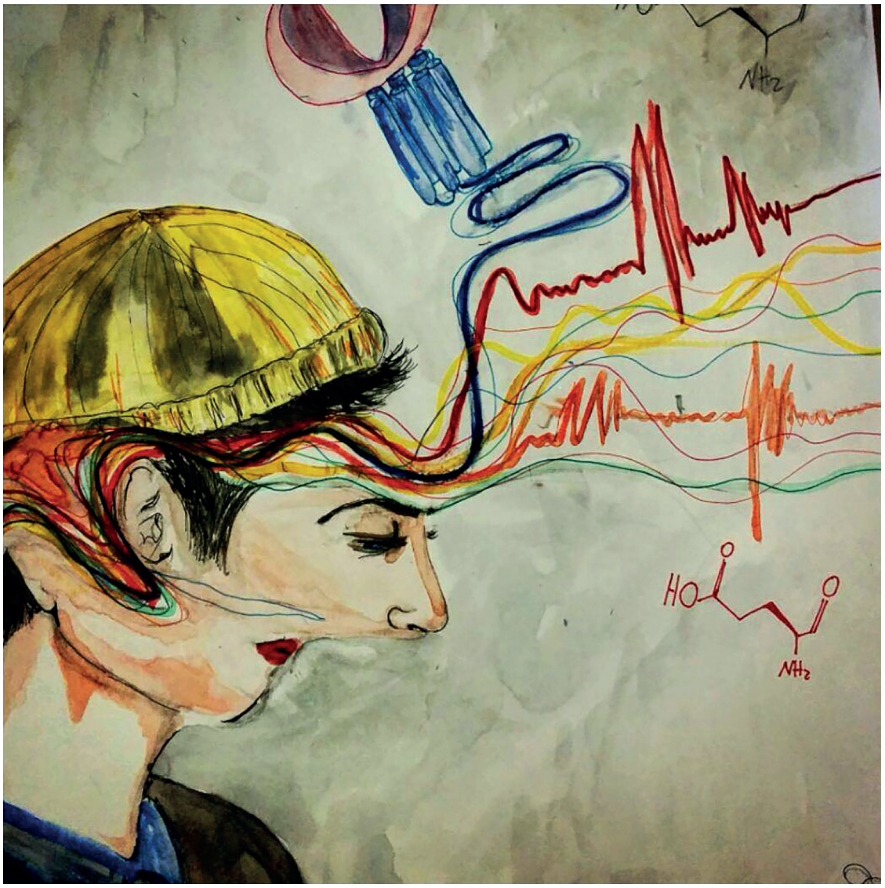
Willott JF, Henry KR. 1976. Roles of anoxia and noise-induced hearing loss in the postictal refractory period for audiogenic seizures in mice. J Comp Physiol Psychol. 90, 373-81.

Willoughby JO, Mackenzie L. 1992. Non convulsive electrocorticographic paroxysms (absence epilepsy) in rat strains. Lab Anim Sci. 42, 551-4.

Wong CG, Scherer SW, Snead OC III, Hampson DR. 2001 Localization of the human mGluR4 gene within an epilepsy susceptibility locus(1). Brain Res Mol Brain 87, 109-16.

Wootten D, Simms J, Miller LJ, Christopoulos A, Sexton PM. 2013. Polar transmembrane interactions drive formation of ligand-specific and signal pathway-biased family B G protein-coupled receptor conformations. Proc Natl Acad Sci U S A. 110, 5211-6.

Zhang CS, Bertaso F, Eulenburg V, Lerner-Natoli M, Herin GA, Bauer L, Bockaert J, Fagni L, Betz H, Scheschonka A. 2008. Knock-in mice lacking the PDZ-ligand motif of mGluR7a show impaired PKC-dependent autoinhibition of glutamate release, spatial working memory deficits, and increased susceptibility to pentylenetetrazol. J Neurosci. 28, 8604-14.



# 2 Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy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# Abstract

We examined the expression and function of mGlu1 receptors in the thalamus of WAG/Rij rats, which develop spontaneous absence seizures at >3 months of age. Reduced mGlu1 receptor mRNA levels were found in a thalamic region comprising the LDVL/LPMR thalamic nuclei (ventrolateral part of the laterodorsal thalamic nucleus/mediorostral part of the lateral posterior thalamic nucleus) of symptomatic 8-month old WAG/Rij rats as compared to pre-symptomatic 2-month old WAG/Rij rats or age-matched non-epileptic control rats. mGlu1 receptor mRNA levels were undetectable in the reticular thalamic nucleus (RTN). Immunoblot and immunohistochemical analysis of mGlu1α receptor protein confirmed the reduction of mGlu1 receptor expression in thalamic relay nuclei of symptomatic WAG/Rij rats. mGlu1 receptor signalling was also reduced in the thalamus of 8-month old WAG/Rij rats, as assessed by measurements of agonist-stimulated polyphosphoinositide hydrolysis in living animals. Moving from these findings, we examined whether pharmacological activation of mGlu1 receptors could affect the epileptic phenotype of WAG/Rij rats. Systemic treatment with the selective mGlu1 receptor enhancer, SYN119 (10 mg/kg, s.c.; corresponding to RO0711401), substantially reduced the incidence of spike and wave discharges (SWDs) in WAG/Rij rats without affecting SWD duration or spontaneous motor activity. In contrast, treatment with the mGlu1 receptor antagonist, JNJ16259685 (2.5 and 5 mg/kg, i.p.), enhanced the incidence of SWDs. These data suggest that absence epilepsy might be associated with a reduction of mGlu1 receptors in the thalamus, and that compounds that amplify the activity of mGlu1 receptors might be developed as novel anti-absence drugs.


**Keywords:** Absence seizures; *"in vivo"* mGlu1 receptors signalling; spike and wave discharges; WAG/Rij rats.

# Introduction

A growing body of evidence suggests a role for metabotropic glutamate (mGlu) receptors in the pathophysiology of absence epilepsy, characterized by spontaneous seizures and spike-wave discharges (SWDs) in the electroencephalogram. mGlu receptors form a family of eight subtypes (mGlu1-8) subdivided into three groups on the basis of their amino acid sequence, pharmacological profile, and transduction mechanisms. Group I includes mGlu1 and mGlu5 receptors, which are coupled to $G_q/G_{11}$ proteins and are predominantly localized in the peripheral portion of postsynaptic densities. Group II includes mGlu2 and mGlu3 receptors, which are coupled to $G_i/G_o$ proteins and are localized in the pre-terminal region of axon terminals, where they negatively regulate neurotransmitter release. Group III includes mGlu4, mGlu6, mGlu7, and mGlu8 receptors, which are also coupled to $G_i/G_o$ proteins in heterologous expression systems. mGlu4, mGlu7, and mGlu8 receptors are presynaptically localized near the active zone of neurotransmitter release; mGlu6 receptors are exclusively expressed by ON bipolar cells of the retina. mGlu3 and mGlu5 receptors are also present in astrocytes (reviewed by Niswender and Conn, 2010). Studies with chemical and genetic models of absence epilepsy have consistently shown that the mGlu4 receptor has a permissive role in the development of absence seizures. Mice with genetic deletion of mGlu4 receptors show abnormalities in excitatory and inhibitory neurotransmission in the corticothalamic network underlying absence seizures (Wang et al., 2005), and are resistant to chemically induced absence seizures (Snead et al., 2000). Wistar Albino Glaxo from Rijswijk (WAG/Rij) rats, which provide a validated genetic rat model of absence epilepsy (Coenen and van Luijtelaar, 2003; van Luijtelaar and Sitnikova, 2006), show an increased incidence of pathological SWDs when treated with the selective mGlu4 receptor enhancer, N-phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxamide (PHCCC) (Ngomba et al., 2008). In humans, the mGlu4 receptor gene is localized at a susceptibility locus for juvenile myoclonic epilepsy (Izzi et al., 2003; Wong et al., 2001), which is characterized by absence seizures in addition to myoclonus and tonic-clonic seizures. Another group-III mGlu receptor, the mGlu7 receptor, is also associated with the development of absence seizures. Mutant mice in which mGlu7 receptors fail to interact with PICK1 (protein interacting with C kinase 1) develop absence seizures (Bertaso et al., 2008; Zhang et al., 2008), as do mice injected with a peptide that disrupts the interaction between mGlu7 receptors and PICK1 (Bertaso et al., 2008). The precise mechanism whereby interaction between mGlu7 receptors and PICK1 restrains SWDs in the corticothalamic network remains to be determined. The role of mGlu2 and mGlu3 receptors in absence seizures is controversial. Pharmacological blockade of mGlu2/3 receptors reduces the incidence of SWDs in WAG/Rij rats, whereas treatment with a selective mGlu2/3 receptor agonist increases absence seizures in these rats (Ngomba et al., 2005). In contrast, the activation of mGlu2/3 receptors reduces the occurrence of absence seizures in *lethargic* mice (Moldrich et al., 2001), these mice show

an epileptic phenotype because of a mutation in the gene encoding the β4 subunit of voltage-sensitive calcium channels (Burgess et al., 1999).

mGlu1 receptors are highly expressed in thalamic relay neurons (Baude et al., 1993; Ferraguti et al., 2008; Godwin et al., 1996; Liu et al., 1998; Martin et al., 1992; Shigemoto et al., 1992; Vidnyanszky et al., 1996), which are part of a thalamocortical loop involved in the generation of absence seizures (reviewed by Blumenfeld, 2005; van Luijtelaar and Sitnikova, 2006). However, evidence linking mGlu1 receptors to the development of absence seizures is still indirect and controversial. Deletion of phospholipase C beta[4] (PLCβ4) in thalamocortical relay nuclei leads to absence seizures in mice (Cheong et al., 2009). PLCβ4 colocalizes with mGlu1 receptors, and mediates mGlu1 receptor signalling in thalamic nuclei (Miyata et al., 2003; Watanabe et al., 1998). The hypothesis that an impaired mGlu1 receptor signalling in the thalamus facilitates the development of absence seizures is not in line with data obtained in lethargic mice, where treatment with orthosteric mGlu1 receptor antagonists reduces the occurrence of SWDs (Burgess et al., 1997; Chapman et al., 1999). We therefore decided to examine in detail the possible link between mGlu1 receptors and spontaneous absence seizures using WAG/Rij rats as a model. We report that symptomatic 8-month old WAG/Rij rats show a reduced expression and signalling of mGlu1 receptors in thalamic nuclei, and that pharmacological activation of mGlu1 receptors with a selective enhancer reduces the incidence of spontaneous absence seizures. Part of these data have been presented in Abstract form (Ngomba et al., 2009).

# Methods

## Drugs

JNJ16259685((3,4-dihydro- 2H-pyranol [2,3-b]quinolin-7yl) (cis-4-methoxycycloexyl) methanone), DHPG (3,5-dihydroxyphenylglycine), MPEP (2,methyl-6-(phenylethynyl)-pyridine), and diazepam were purchased from Tocris Cookson Ltd. (Bristol, UK); SYN119 (9H-xanthene-9-carboxylix acid (4-trifluoromethyl-oxazol-2-yl) amide, corresponding to RO0711401, was kindly provided by Synosia Therapeutics Ltd (Basel, Switzerland). JNJ16259685 was dissolved in saline containing 10% hydroxypropyl-b-cyclodextrin, and injected i.p.. SYN119 was dissolved in peanut oil, and injected s.c..

## Animals

Male WAG/Rij and ACI (Augouti Copenhagen Irish) rats were kept under environmentally controlled conditions (ambient temperature = 22 °C, humidity = 40%) in a room with reversed light-dark cycle (light on from 8:00 p.m. to 8:00 a.m.), with food and water ad libitum. Experiments were carried out during the dark phase of the cycle in which WAG/Rij rats have the largest amount of SWDs (van Luijtelaar and Coenen, 1988). All animals were handled prior to EEG registrations. For biochemical studies, age matched ACI rats

were used as controls; rats of this strain have no or at least very few SWDs and in all cases they have much fewer SWDs than do WAG/Rij rats of the same age (Inoue et al., 1990; Schridde & van Luijtelaar, 2004). Of all inbred rats investigated, ACI rats have the lowest number of SWDs (Inoue et al., 1990). Therefore, ACI rats are commonly used as controls in experiments with WAG/Rij rats (Ngomba et al., 2008; van Rijn et al., 2010). We used both strains of rats at 2 or 8 months of age. WAG/Rij rats of 2 months of age do not show SWDs as yet, and, therefore, are considered "presymptomatic". This study was performed in accordance with the guidelines of the European Community for the use of experimental animals. Approval of the local ethics committee for animal studies was obtained.

## Analysis of mGlu1 receptor

### In situ hybridization of mGlu1 receptor mRNA

In situ hybridization was carried out as described previously (Simonyi et al., 2005). Twelve micron coronal sections were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 5 min, rinsed in PBS, and soaked in 0.25% acetic anhydride in 0.1M triethanolamine hydrochloride/0.9% NaCl (pH 8.0) for 10 min. Sections were rinsed in 2 X SSC (300 mM NaCl/30 mM sodium citrate), dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated to 95% ethanol and air-dried. Fifty microliters of hybridization buffer was applied to each slide, covered with a coverslip, and incubated at 42°C overnight. The hybridization buffer contained 50% formamide, 4 X SSC, transfer RNA (250 µg/ml), sheared, single-stranded salmon sperm DNA (100 µg/ml), 1 X Denhardt's solution (0.02% BSA, Ficoll, and polyvinylpyrrolidone), 10% (w/v) dextran sulfate (MW 500,000), 50 mM DTT and 1 X $10^6$ cpm probe. Probes were 3' end-labeled by terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN) with $^{35}$S-dATP (NEN, Boston, MA). The probe for metabotropic glutamate receptor 1 subtype was the same as in our earlier publications (Simonyi et al., 2005). The oligomer sequence of the mGlu1 receptor probe was complementary to cDNA sequence 1450-1495 (Masu et al., 1991) and it recognizes all the splice variants of mGlu1 receptor. After hybridization, coverslips were removed in 1 X SSC. Slides were washed in 1 X SSC (2 mM DTT) at 55°C for 4 X 15 min. Following two 30 min rinses in 1 X SSC at room temperature, the tissues were dipped in distilled water, immersed in 70% ethanol and air-dried. All tissues to be evaluated by a single statistical test were included in the same hybridization. Slides were held against KODAK BIOMAX MR films with standards (American Radiolabeled Chemicals Inc., St. Louis, MO) in X-ray cassettes. Microdensitometry was performed on the signal over different brain regions using the BIOQUANT True Color Windows 95 software version 2.50, as previously described (Simonyi et al., 2005). [$^{14}$C]-Microscale standards were used to construct calibration curves and quantitate signals in nCi/g tissue equivalents. The average density measured from experimental regions fell within the linear range of the standards. Background signal was subtracted from all measurements. Values were averaged from the analysis of two sections for each animal (5-6 per group) before being evaluated for

statistical significance. The Paxinos and Watson atlas (2005) was used for identification of brain nuclei. Sections were examined by two independent operators who were not aware of the experimental protocol.

**Western blot analysis of mGlu1 receptors**

Male WAG/Rij and matched control ACI rats of 2 or 8 months of age were anesthetized with ether, decapitated and brains were rapidly removed from the skulls and frozen. Brains from each rat were coded and codes were released after Western blot analysis. Brains were frontally dissected on a cryostat, and a thalamic portion containing the reticular thalamic nucleus (RTN) or another portion containing all other thalamic nuclei (referred to as "ventrobasal" – VB – thalamus) were manually dissected out under the guide of the Paxinos and Watson atlas (2005). To examine the specificity of the mGlu1α antibody, we also performed immunoblot analysis on brain regions dissected from 2-month old male homozygous mutant mice carrying the *cervelet-4* (*crv4*) mutation or age-matched BALB/c control mice. Homozygous *crv4* mice are characterized by the absence of the mGlu1 receptor protein due to the insertion of a 190-bp LTR fragment in intron 4 of the *Grm1* gene (Conti et al., 2006). Tissue was homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. After sonication, 2 µl of total extracts were used for protein determinations. One hundred micrograms of protein extract were resuspended in SDS-bromophenol blue reducing buffer with 40 mM DTT. Western blot analyses were carried out by loading 35 µg of total proteins per lane into 8% SDS polyacrylamide gels, which were electroblotted on immunblot PVDF membranes (BioRad, Milano, Italy). The PVDF membranes were blocked overnight in TBS-T buffer (100 mM Tris-HCl; 0.9% NaCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated for 1 h at room temperature with rabbit polyclonal anti-mGlu1α antibodies (1:500, Upstate Biotechnology, Lake Placid, NY) and a mouse monoclonal antibody to label β-actin (1:100.000, Sigma, St. Louis, MO). Filters were washed with TBS-T buffer and then incubated for 1 hour with secondary antibodies (peroxidase-coupled anti-rabbit or anti-mouse; 1:7000; Amersham, Piscataway, NJ). Immunoreactivity was revealed by ECL.

**Immunohistochemistry**

Brains from WAG/Rij rats, ACI rats, homozygous *crv4* mice, and BALB/c control mice were fixed in Carnoi, embedded in paraffin, and sectioned at 10 µm. Subsequently, deparaffinized sections were treated in 10 mM, pH 6.0, citrate buffer, and heated by microwave for 10 min for antigen retrieval, and immersed in 3% $H_2O_2$ for 10 min to quench endogenous peroxidase activity. The slides were allowed to cool for 20 min in the same solution at room temperature and then washed in TBS. The antibodies were dissolved in TBS containing 2% normal goat serum. Sections were preincubated for 1 hour with normal goat serum (Vector Laboratories, Burlingame, CA) and with polyclonal anti-mGlu1α

antibodies (Upstate Biotechnology; 1:50), and then for 1 hour with secondary biotinylated anti-goat antibodies (1:200; Vector Laboratories). 3,3-Diaminobenzidine tetrachloride was used for detection (ABC Elite kit; Vector Laboratories). Control staining was performed without the primary antibodies. Intensity of mGlu1α immunoreactivity in three thalamic regions (see Fig. 3b) was quantified by measuring the relative optical densities. Images were acquired at low magnification (2.5x) and the densitometric analysis was performed by assessing the intensity of the background values (i.e. the optical density measured in unlabeled areas present in the section, such as corpus callosum).
The optical density values were obtained from the dorsal complex , D = [lateral posterior thalamic nucleus, mediorostral part (LPMR) + laterodorsal thalamic nucleus, ventrolateral part (LDVL) + laterodorsal thalamic nucleus, dorsomedial part (LDDM)], the medial complex, M =[posterior thalamic nuclear group (Po) + the internal ventroposteromedial part (VPM)] and lateral complex, L = [ventroposterolateral (VPL) nucleus] (see Fig. 3b).



**In vivo experiments**

***Measurement of DHPG-stimulated polyphosphoinositide (PI) hydrolysis in living rats***

ACI and WAG/Rij rats were anesthetized with ketamine (100 mg/kg) plus xylazine (10 mg/kg) and injected with [*myo*-$^{3}$H]inositol (2 µCi/5 µl/2 min, i.c.v.). Twenty-four hours later, rats were treated with lithium ions (administered as LiCl, 10 mmol/kg, s.c.) to inhibit the conversion of inositol monophosphate (InsP) into free inositol. The mGlu1/5 receptor agonist, DHPG (500 nmol/5 µl saline containing 50% dimethyl sulfoxide), was injected i.c.v., 1 hour after LiCl injection. Control rats were injected with the vehicle alone. The selective mGlu5 receptor antagonist, MPEP (10 mg/kg), and diazepam (10 mg/kg) – used as an anti-convulsant - were injected i.p. 30 min before DHPG. Rats were killed 1 hour after treatment with DHPG or vehicle. The thalamus was quickly removed and stored at -80°C. On the day of the assay, tissue was sonicated in 1.25 ml of water containing 10 mM LiCl. After centrifugation at 10,000 x *g* for 20 min, the [$^{3}$H]InsP present in the supernatant was separated by anion exchange chromatography in 10 ml columns containing 1.5 ml of Dowex 1-X-8 resin (formate form, 100–200 mesh; Bio-Rad). Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP (Nicoletti et al., 1986). Total radioactivity in the brain regions was determined by counting a 100 µl aliquot of the whole homogenate.

***EEG recordings***

Male WAG/Rij rats (8-month old) with a mean body weight of 390 ± 17 g, were used. Animals were individually housed in Macrolon cages in a room illuminated with white lights from 8:00 p.m. to 8:00 a.m.. A permanent cortical tripolar electrode set was implanted under complete isoflurane anesthesia, one electrode into the frontal region

(coordinates with the skull surface flat and from bregma zero-zero, AP +2.0; L -2.5), and the other in the parietal region (A -6.0; L -4.0) (Paxinos and Watson, 2005). The ground electrode was placed over the cerebellum. After the surgery, animals were allowed to recover for two weeks. Rats were put into transparent recording cages, connected to an EEG cable which allowed free movement, and habituated to the experimental conditions for 12 h. The EEG was filtered (only frequencies between 0.1 and 100 Hz, were allowed to pass, digitalized with a sample frequency of 512 Hz, and stored for an off-line analysis using Windaq system (DATAQ Instruments, Akron, OH, USA). Baseline EEG was recorded during the dark period (9:00-10:00 a.m) for 1 hour prior to injection and thereafter, following injection of the selective mGlu1 receptor antagonist, JNJ16259685 (2.5 or 5 mg/kg, i.p.) and the mGlu1 receptor positive allosteric modulator (PAM), SYN119 (3 or 10mg/kg, s.c.), or vehicle. The post injection EEG was registered during the next 6 hours. SWDs were marked at visual inspection based on commonly used criteria: trains of sharp spikes and slow waves lasting minimally 1 sec, the amplitude of the spikes minimally twice the background, frequency of the SWDs between 7 and 10 Hz and an asymmetric appearance of the SWDs (van Luijtelaar and Coenen, 1986).

***Recording of spontaneous motor activity***

As previously reported by van Rijn et al. (2010), spontaneous motor activity in symptomatic WAG/Rij rats was recorded by means of an analogic passive infrared detector (PIR) (Luna PR, Rokonet Electronics LTD, Rishon Le Tzion, Israel). The analog signal was digitalized simultaneously with the EEG signal. Movements were quantified by calculating the mean of the absolute value of the PIR signal per hour. The value of the post injection hours of each separate animal was related to the mean baseline value of all animals.

# Results

## Reduced expression of mGlu1 receptors in the ventrobasal thalamus of symptomatic WAG/Rij rats

We examined the expression of mGlu1 receptor mRNA by *in situ* hybridization using a pan-probe that detects all receptor variants. Data obtained in the somatosensory cortex, thalamic relay nuclei, dorsal striatum, and hippocampal dentate gyrus (DG), CA1 and CA3 regions are shown in Table 1. Background level hybridization signal was observed in the reticular thalamic (RT) nucleus (not shown), whereas intense labelling was detected in all thalamic relay nuclei, in agreement with previous studies (Shigemoto and Mizuno, 2000). mGlu1 receptor mRNA levels did not change in any brain region between control rats at 2 and 8 months of age and between control rats and pre-symptomatic WAG/Rij rats at 2 months of age. mGlu1 mRNA levels were lower in LDVL/LPMR thalamic nuclei (ventrolateral part of the laterodorsal thalamic nucleus/mediorostral part of the lateral posterior

**Table 1** In situ hybridization analysis of mGlu1 receptor mRNA levels in selected brain regions of controls (ACI rats and WAG/Rij rats at 2 and 8 months of age.

| | 2-month old | | 8-month old | |
|---|---|---|---|---|
| | ACI | WAG/Rij | ACI | WAG/Rij |
| Somatosensory cortex | 25.5 ± 2.0 | 29.0 ± 3.2 | 29.8 ± 1.1 | 27.2 ± 1.5 |
| LDVL and LPMR | 62.0 ± 1.2 | 57.0 ± 1.0 | 62.0 ± 2.3 | 52.0 ± 1.5[a] |
| VPL | 61.2 ± 1.5 | 61.0 ± 1.1 | 63.2 ± 1.0 | 61.0 ± 3.0 |
| Striatum | 39.0 ± 1.6 | 44.8 ± 3.3 | 40.8 ± 1.4 | 36.2 ± 1.6[b] |
| CA1 | 70.0 ± 3.9 | 80.0 ± 4.6 | 79.0 ± 4.5 | 73.0 ± 6.9 |
| CA3 | 183 ± 8.6 | 180 ± 11.8 | 177 ± 4.4 | 177 ± 6.2 |
| Dentate gyrus | 240 ± 7.9 | 247 ± 9.7 | 235 ± 10.4 | 218 ± 7.4 |

Data are expressed as means of nCi/g tissue ± SEM from 5–6 animals per group. Data were analyzed by two-way ANOVA with strain and age as factors followed by Bonferroni's post test. For LVDL and LPMR, there was significant main effect of strain [$F(1.19) = 15.87$, $p = 0.0008$.

[a] $p < 0.01$ ACI, 8-month old, vs. WAG/Rij, 8-month old]. For the striatum there was a significant interaction [$F(1.18) = 7.18$, $p = 0.0153$.

[b] $p < 0.05$ WAG/Rij, 2-month old, vs. WAG/Rij, 8-month old].

thalamic nucleus) of symptomatic 8-month old WAG/Rij rats as compared to either age-matched control rats or pre-symptomatic 2-month old WAG/Rij rats. No changes in mGlu1 receptor mRNA levels were found in the ventroposterolateral (VPL) thalamic nucleus, somatosensory cortex, and hippocampal subregions of 8-month old WAG/Rij rats as compared with all other groups of rats (Table 1).

We examined the expression of mGlu1α receptor protein using a polyclonal antibody that recognizes the C-terminus domain of the receptor. The specificity of the antibody has been tested by immunoblotting and immunohistochemistry using brain tissue from wild-type mice or from cervelet-4 (*crv4*), homozygous mutant mice lacking the mGlu1 receptor protein (Conti et al., 2006). Immunoblot analysis showed a prominent expression of mGlu1α receptors in the cerebellum and thalamus of wild-type mice, as shown by a band of about 140 kDa, which likely corresponds to receptor monomers, and a higher molecular size band, which may correspond to receptor dimers. Receptor expression was lower in the olfactory bulb and cerebral cortex of wild-type mice, as expected (Shigemoto and Mizuno, 2000). The receptor was virtually absent in all brain regions of *crv4* mice (Fig. 1A). These data were paralleled by immunohistochemical analysis, which showed a prominent mGlu1α receptor immunostaining in the cerebellum and thalamus of wild-type mice, and no staining in *crv4* mice, with the exception of some staining in the external portion of the cerebellar cortex, which may be non-specific (Fig. 1B). Expression data of the mGlu1α receptor in the ventrobasal thalamus of WAG/Rij rats and their controls are shown in Fig. 2. Immunoblot analysis showed that expression of mGlu1α receptor was lower in the thalamus (dissected out as in Fig. 2A) of 8-month old WAG/Rij rats, as compared to either

2-month old WAG/Rij rats and 8-month old control rats (Fig. 2B,C). Expression of mGlu1α receptors was nearly undetectable in a more lateral portion of the thalamus cut inside-out from the internal border of the RTN (not shown). Immunohistochemical analysis (Fig. 3A) showed that the reduced expression of mGlu1α receptors observed in 8-month old sympthomatic WAG/Rij rats was restricted to portions of the thalamus comprising LDVL and LPMR (D = dorsal complex), or the posterior thalamic nuclear group (Po) and the internal part of the ventroposteromedial (VPM) nucleus (M = medial complex). The intensity of immunostaining was not different in a more external portion of the thalamus including the ventroposterolateral (VPL) nucleus (L = lateral complex) (Fig. 3B,C).

## Reduced mGlu1 receptor signalling in the thalamus of symptomatic WAG/Rij rats

To examine whether mGlu1 receptor function was defective in the thalamus of symptomatic WAG/Rij rats, we used an *in vivo* method that allows measurements of agonist-stimulated PI hydrolysis after incorporation of [$^3$H]inositol into the phospholipids of living rats (Molinaro et al., 2009). Eight-month old WAG/Rij rats and their age-matched controls were injected i.c.v. with [$^3$H]inositol and treated, 24 hours later, with litium ions to inhibit the conversion of [$^3$H]InsP into free [$^3$H]inositol. Afterwards, rats were treated

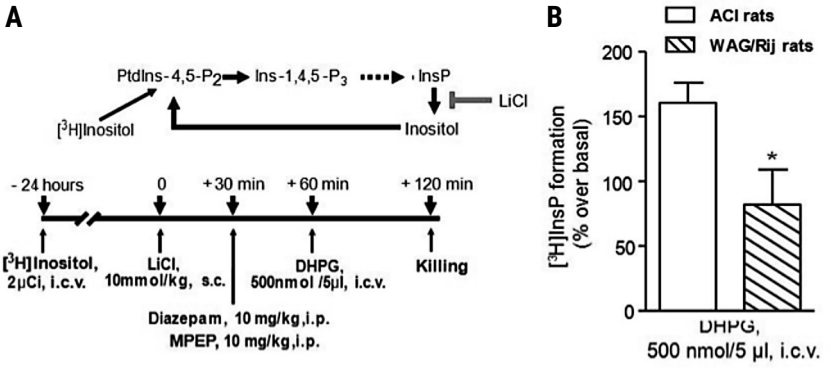


**Figure 1** Reduced mGlu1 receptor signalling in the thalamus of symptomatic WAG/Rij rats. In vivo measurements of PI hydrolysis in the thalamus of 8-month old WAG/Rij rats and age-matched controls (ACI rats) was carried out as outlined in (A). PtdIns-4,5,$P_2$ = phosphatidylinositol-4,5-bisphosphate; Ins-1,4,5-P$^3$ = inositol-1,4,5-trisphosphate; InsP = inositolmonophosphate; hrs = hours. (B) DHPG-stimulated PI hydrolysis in the thalamus of WAG/Rij (n = 5 animals per group). *$p < 0.05$ (Student's t test) vs. values obtained in controls rats. Basal values of [$^3$H] InsP formation normalized by the amount of total radioactivity in each sample were 1.66 ± 0.05 and 1.64 ± 0.12 (means ± S.E.M.) in the thalamus of ACI and WAG/Rij rats, respectively.

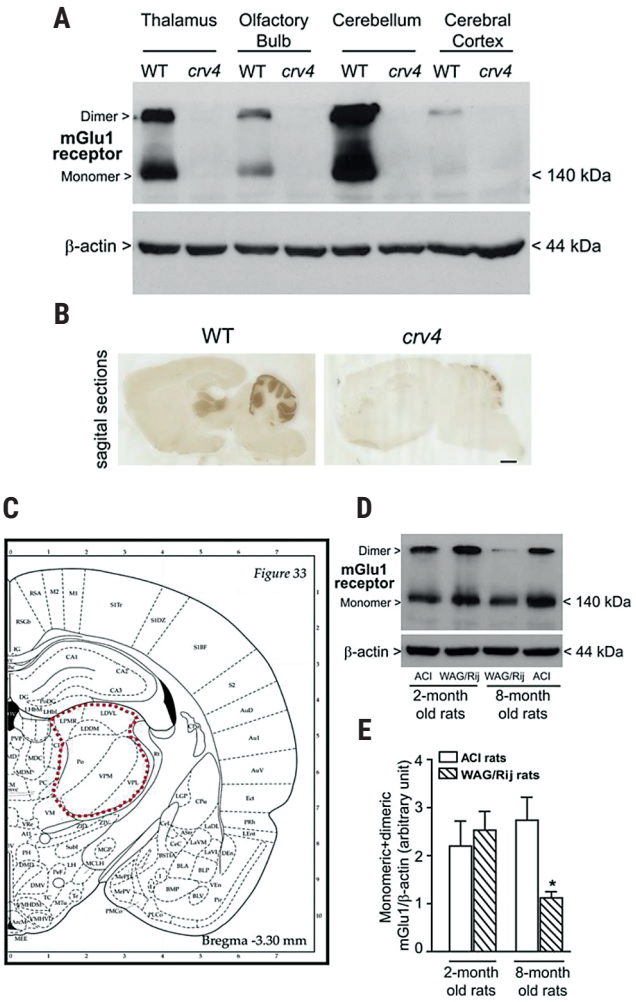


**Figure 2** Reduced expression of mGlu1a receptors in the thalamus of symptomatic WAG/Rij rats: immunoblot analysis. Specificity of the antibody used for the detection of mGlu1α receptors is shown in A-B. Western blot analysis of mGlu1a receptors in different regions of wild-type (WT) BALB/c mice and homozygous crv4 mice is shown in (A). mGlu1a immunostaining in a sagittal section of the whole brain of WT and crv4 mice is shown in (B). Scale bar ¼ 1 mm. The portion of the thalamus dissected for immunoblot analysis is shown in (C). Note that the RTN is excluded from this area. A representative immunoblot showing the expression of mGlu1a receptors in the thalamus of 2- and 8-month old WAG/Rij rats and age-matched controls (ACI rats) is shown in (D). Densitometric analysis is shown in (E), where values are means ± S.E.M. of 5e6 determinations. *p < 0.05 (One-way ANOVA þ Fisher's PLSD) vs. all other values.

systemically with a cocktail of MPEP (to prevent the activation of mGlu5 receptors) and diazepam (to prevent convulsive seizures), followed by i.c.v, injection of the mGlu1/5 receptor agonist, DHPG (Fig. 4A). Treatment with DHPG increased PI hydrolysis by >2.5 fold in the thalamus of 8-month old ACI control rats, and only by 80% in 8-month old WAG/Rij rats (Fig. 4B). Thus, mGlu1-receptor mediated PI hydrolysis was reduced by about 50% in the thalamus of symptomatic WAG/Rij rats as compared to age-matched controls.

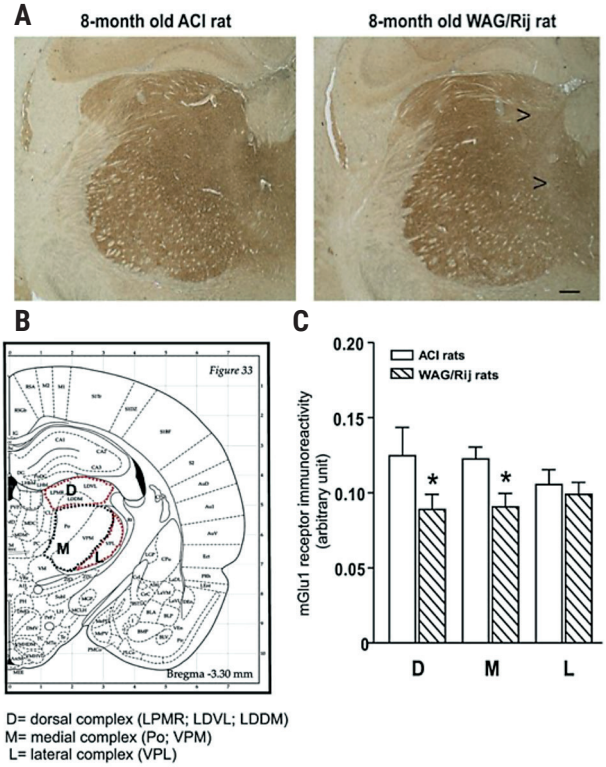


**Figure 3** Reduced expression of mGlu1a receptors in the thalamus of symptomatic WAG/Rij rats: immunohistochemical analysis. A representative mGlu1a immunostaining in the thalamus of 8-month old WAG/Rij rats and age-matched controls (ACI rats) is shown in (A), where arrowheads indicate the regions of reduced immunolabeling in WAG/Rij rats (scale bar = 250 mm). The thalamic regions selected for densitometric analysis are shown in (B), and densitometric data are shown in (C). Values are means ± S.E.M. and were calculated from the average of 8 coronal sections for each animal (n = 6 animals per group). *$p < 0.05$ (Student's t test) vs. the corresponding values obtained in control rats.

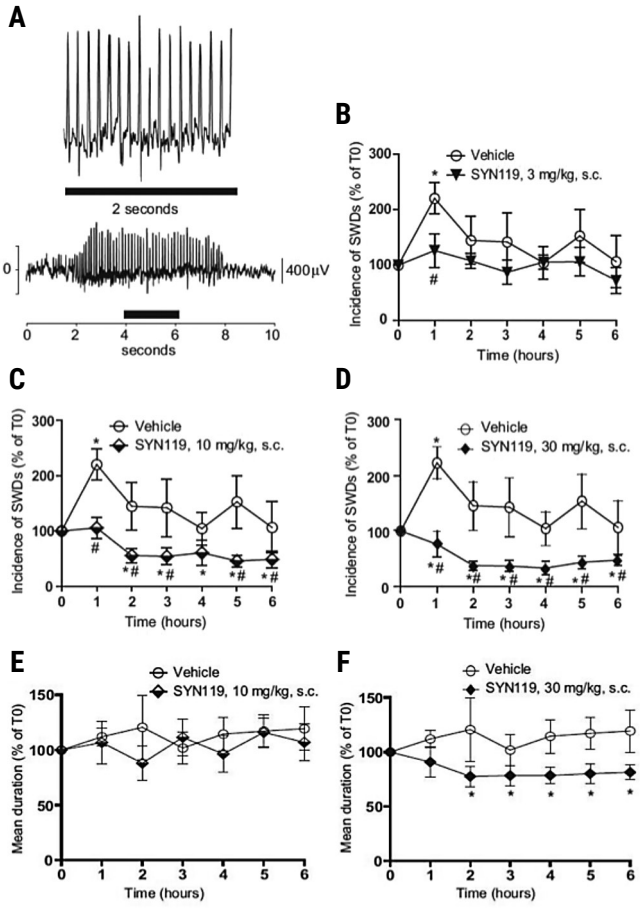


**Figure 4** Pharmacological activation of mGlu1 receptors reduces the incidence of SWDs in WAG/Rij rats. A representative EEG recording of SWDs is shown in (A) where segments of recording at an expanded timescale is marked by the black bars. The incidence of SWDs in 8-month old WAG/Rij rats treated systemically with vehicle or two different doses of the mGlu1 receptor PAM, SYN119, is shown in (B), (C) and (D). Mean duration of SWDs in WAG/Rij rats treated with vehicle or with the higher doses of SYN119 (10 mg/kg and 30 mg/kg) are shown in (E) and (F). Values are means ± S.E.M. of 6-8 animals. $p < 0.05$ (ANOVA for repeated measures followed by Dunn's t test) vs. the corresponding values at baseline (T0) (*) or vs. the corresponding values obtained in WAG/Rij rats treated with vehicle (#).

## Pharmacological activation of mGlu1 receptor reduces the incidence of SWDs in WAG/Rij rats

To examine the impact of mGlu1 receptors on the occurrence of spontaneous absence seizures, 8-month old WAG/Rij rats were treated systemically with compound SYN119 (3 or 10 mg/kg, s.c., corresponding to RO0711401), which behaves as a selective PAM of mGlu1 receptors (Vieira et al., 2009). By definition, a PAM exclusively amplifies receptors that are activated by endogenous glutamate (or by another orthosteric agonist), thus acting in a activity-dependent fashion. We used SYN119 at doses that are known to be centrally active (Fazio et al., 2008). EEG was recorded in WAG/Rij rats for 1 hour at baseline (T0) and then for 6 hours following SYN119 administration. A typical EEG recording of SWDs in WAG/Rij rats is shown in (Fig. 5A). Treatment with vehicle invariably led to an increase in the incidence of SWDs in the first hour, followed by a reduction afterwards.

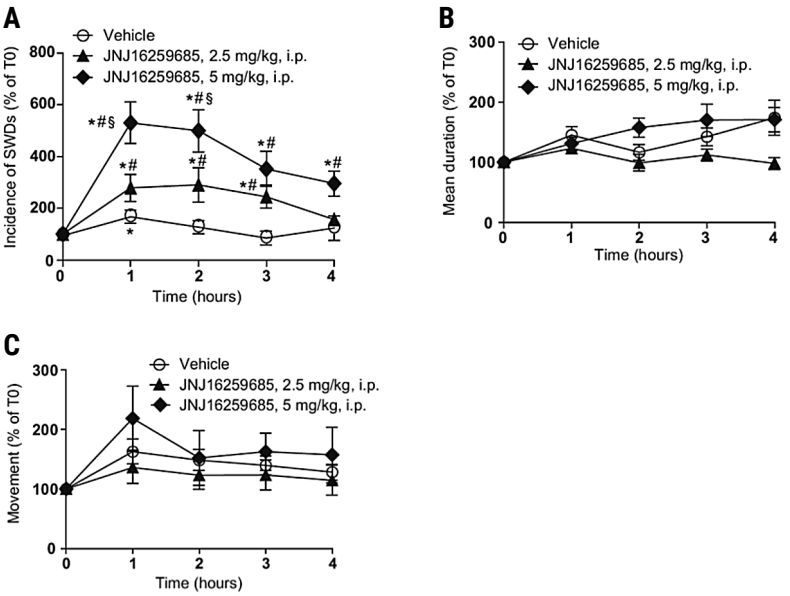


**Figure 5** Pharmacological inhibition of mGlu1 receptors enhances the incidence of SWDs in WAG/Rij rats. The incidence of SWDs in 8-month old WAG/Rij rats treated systemically with vehicle or two different doses of the mGlu1 receptor NAM, JNJ16259685 (JNJ) is shown in (A). Values are means ± S.E.M. of 8 animals per group. $p < 0.05$ (ANOVA for repeated measures followed by Dunn's t test) vs. the corre- sponding values at baseline (T0) (*); vs. the corresponding values obtained in rats treated with vehicle (#); or vs. the corresponding values obtained in rats treated with lower dose of JNJ16259685 (x). Mean duration of SWDs and spontaneous motor activity in WAG/Rij rats treated with vehicle or both doses of JNJ16259685 are shown in (B) and (C), respectively.

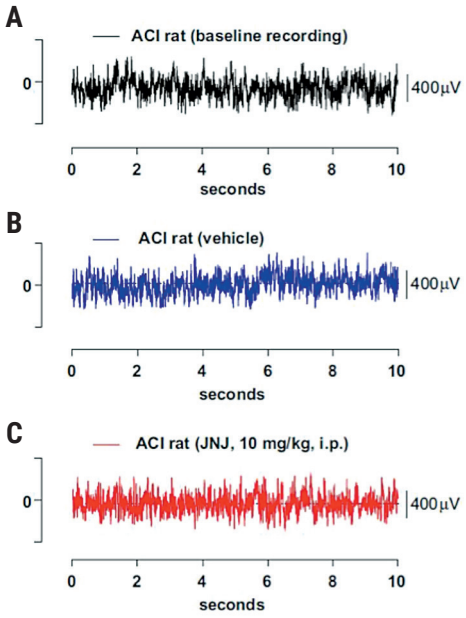


**Figure 6** Pharmacological inhibition of mGlu1 receptors does not alter baseline EEG recordings in ACI rats. Representative EEG recordings from ACI rats shown in (AeC); The EEG segments were extracted from recordings during baseline (A) and from the second hours post injection of either vehicle (B) or 10 mg/kg, i.p. of JNJ16259685 (C).

This increase might reflect the stress associated with the injection as corticosteroids tend to enhance SWDs (Schridde and van Luijtelaar, 2004). Treatment with the highest dose of SYN119 (10 mg/kg) significantly reduced the incidence of SWDs at all times. After the first hour, the incidence of SWDs was significantly lower than that recorded at baseline (Fig. 5B,C). Treatment with the lowest dose of SYN119 (3 mg/kg) significantly reduced the incidence of SWDs only during the first hour (Fig. 5B). This indicated that a dose of SYN119 of 10 mg/kg was required for a substantial reduction in the incidence of absence seizures in WAG/Rij rats, whereas the dose of 3 mg/kg of SYN119 could only abolish the early stress-related increase in the incidence of SWDs. At the most effective dose (10 mg/kg) SYN119 had no effect on the mean duration of single SWD episodes (Fig. 5D), and showed only marginal and non-significant effects on spontaneous motor activity (Fig. 5E). To further demonstrate a protective role for mGlu1 receptors against spontaneous absence seizurers, additional groups of 8-month old WAG/Rij rats were systemically injected with compound JNJ16259685 (2.5 or 5 mg/kg, i.p.), which behaves as a selective negative allosteric modulator (NAM) of mGlu1 receptors. Treatment with JNJ16259685 increased the incidence of SWDs in a

dose-dependent manner. The incidence of SWDs was increased by as much as 5 fold in the first 2 hours after treatment with the highest dose of JNJ16259685 (Fig. 6A). Again, treatment with the mGlu1 receptor NAM did not change neither the mean duration of SWDs (Fig. 6B) nor the spontaneous motor activity (Fig. 6C) in WAG/Rij rats.

# Discussion

WAG/Rij rats are widely used as a genetic animal model of absence epilepsy. After 3 months of age, these rats develop generalized bilateral SWDs (frequency: 7-9 Hz; duration of 1-30 sec) associated with behavioural manifestations that mimic those of the human petit male attack (Coenen and van Luijtelaar, 2003; van Luijtelaar and Sitnikova, 2006). The incidence of SWDs in WAG/Rij rats is reduced by typical anti-absence drugs, such as ethosuximide and valproate, and is increased by drugs that are known to aggrevate absence epilepsy, such as phenytoin, carbamazepine, and vigabatrine (Bouwman et al., 2007; Coenen and van Luijtelaar, 2003; Peeters et al., 1989). Thus, this model is particularly helpful for the study of the pathophysiology of absence seizures, and has predictive value for the clinical development of new anti-absence drugs. We have shown here that symptomatic (8-month old) WAG/Rij rats show a reduced expression and function of mGlu1 receptors in the thalamus as compared to non-epileptic control rats, and respond to the amplification of residual mGlu1 receptors with a selective PAM with a reduced incidence of absence seizures. In contrast, selective pharmacological blockade of mGlu1 receptors increased the incidence of absence seizures. Remarkably, we have demonstrated the reduction of mGlu1 receptor signalling in the thalamus of WAG/Rij rats using a method that allows the detection of agonist-stimulated PI hydrolysis in living animals (Molinaro et al., 2009). Rats were injected i.c.v. with radioactive inositol, and treated with DHPG and MPEP to selectively activate mGlu1 receptors. This method is highly reliable and avoids the bias of the slice preparation for the evaluation of receptor signalling. The reduction of mGlu1-receptor stimulated PI hydrolysis found in the thalamus of WAG/Rij rats is in nice agreement with the evidence that deletion of PLCβ4 (the enzyme activated by mGlu1 receptors) in thalamic relay nuclei leads to absence seizures (see Introduction and references therein). Taken together, our data strongly suggest that mGlu1 receptors have a protective function against absence seizures and that WAG/Rij rats develop absence seizures because of an age-dependent reduction in the expression and function of thalamic mGlu1 receptors. The mGlu1 receptor PAM may relieve absence seizures by amplifying the activation of residual thalamic mGlu1 receptors. The advantage of the PAM is that it recruits only those mGlu1 receptors that are activated by the endogenous glutamate, thereby acting in an activity-dependent manner.

Absence seizures are generated within a cortico-thalamo-cortical loop that comprises ventrobasal (VB) thalamic relay neurons, the triggering zone in the somatosensory cortex

(Meeren et al., 2002), cortical pyramidal neurons and interneurons, and inhibitory neurons of the reticular thalamic (RT) nucleus and their anatomical connections. The cortex (layer V and VI) send excitatory glutamatergic projections to the RTN and ventrobasal complex, at his turn VB thalamic neurons send excitatory glutamatergic projections to cortical pyramidal neurons and RTN, RT neurons send inhibitory GABAergic connections to VB thalamic neurons but not to the cortex. RT neurons are also connected to each other *via* inhibitory GABAergic synapses and gap junctions (Blumenfeld, 2005; van Luijtelaar and Sitnikova, 2006; Zhang and Jones, 2004). Thalamocortical neurons produce two distinct firing patterns that are thought to reflect the status of signal transmission from the thalamus to the cortex. Tonic firing is believed to represent a relay mode of afferent sensory signals to the cortex (McCormick and von Krosigk, 1992). On the contrary, low-threshold burst firing has been related to drowsy/sleeps states or decrease of consciousness associated with absence seizures (Crunelli and Leresche, 2002; Yu and Blumenfeld, 2009). At least two splice variants of mGlu1 receptors, mGlu1α and mGlu1αreceptors (Ferraguti et al., 2008), are present at high levels on dendrites of thalamic relay neurons, postsynaptic to axon terminals originating from cortical layer VI neurons (Baude et al., 1993; Godwin et al 1996; Liu et al., 1998; Martin et al., 1992; Shigemoto et al., 1992; Vidnyanszky et al., 1996). Activation of mGlu1 receptors in thalamic relay neurones causes a slow depolarising response mediated by the reduction of a potassium conductance, and may participate in sensory processing by mediating cortical inputs back into the thalamus (Crunelli et al., 2002; Hughes et al. 2002; Reichova and Sherman 2004; Rivadulla et al., 2002; Salt and Turner, 1998; Turner and Salt 2000). Thus, mGlu1 receptors are critically localized to regulate the firing rate and oscillatory properties of thalamic relay neurons. Neurons of the RT nucleus do not express mGlu1 receptor mRNA (Shigemoto et al., 1992), whereas, in the cortex, postsynaptic mGlu1 receptors are found in GABAergic interneurons, but not in pyramidal cells (Stinehelfer et al., 2000). The role for cortical mGlu1 receptors in the overall activity of the thalacocortical network remains to be examined.

The abnormal hypersychronous oscillatory activity of thalamic relay neurons that underlies absence seizures is sustained by a pathological activity of T-type voltage-sensitive $Ca^{2+}$ channels (VSCCs), which recover from inhibition in the hyperpolarizing milieu generated by the GABAergic input from RT projection neurons (reviewed by Blumenfeld, 2005). Interestingly, recent evidence indicates that mGlu1 receptors are functionally coupled to T-type VSCCs in both heterologous expression systems and dendritic spines of Purkinje cells (Hildebrand et al., 2009). In recombinant expression systems, mGlu1 receptor modulation is subtype-specific, potentiating $Ca_v3.1$ and $Ca_v3.2$ isoforms while inhibiting $Ca_v3.3$ T-type channels (Hildebrand et al., 2009). Inhibition of $Ca_v3.3$ T-type VSSCs is shared by muscarinic receptors coupled to $Gq/G_{11}$ protein, and appears to involve the βγ subunits of the G protein (Hildebrand et al., 2007; 2009). The possibility that mGlu1 receptors modulate T-type VSCCs in thalamic relay neurons is attractive and warrants further investigation. A functional coupling between mGlu1 receptors and T-type VSCCs in the

thalamus might contribute to explain the difference between our data obtained in WAG/Rij rats and data obtained by Burgess et al. (1997) and Chapman et al. (1999) in lethargic mice, which respond to treatment with orthosteric mGlu1 receptor antagonists with a reduction of absence seizures. Lethargic mice have a mutated β4 subunit of VSCCs (Burgess et al., 1999), which might affect the modulation of T-type VSCCs by mGlu1 receptors in thalamic relay neurons.

Subtype-selective ligands of mGlu receptors are under clinical development for the treatment of a variety of neurological and psychiatric disorders (reviewed by Nieswender and Conn, 2010). Phase I and II clinical studies suggest that mGlu receptor ligands are in general well tolerated and devoid of the CNS adverse effects that are typically seen with ionotropic glutamate receptor antagonists. Thus, in principle, mGlu1 receptor PAMs might be developed for the treatment of patients with absence epilepsy that are refractory to conventional medication. However, it should be highlighted that mGlu1 receptors mediate synaptic transmission and plasticity at the synapse between parallel fibres and Purkinje cells in the cerebellar cortex, and genetic deletion or blockade of mGlu1 receptors causes ataxia and other cerebellar motor symptoms (Aiba et al., 1994; Marignier et al., 2010; Sillevis Smitt et al., 2000). Although we never observed motor incoordination in WAG/Rij rats treated with the mGlu1 receptor PAM, we cannot exclude that a chronic activation of mGlu1 receptors in Purkinje cells may disrupt cerebellar function and motor learning.

## Acknowledgements

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# References


Aiba, A., Kano, M., Chen, C., Stanton, M.E., Fox, G.D., Herrup, K., Zwingman, T.A., Tonegawa, S., 1994. Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell. 79, 377-88.

Baude, A., Nusser, Z., Roberts, J.D., Mulvihill, E., McIlhinney, R.A., Somogyi, P., 1993. The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron. 11, 771-87.

Bertaso, F., Zhang, C., Scheschonka, A., de Bock, F., Fontanaud, P., Marin, P., Huganir, R.L., Betz, H., Bockaert, J., Fagni L, Lerner-Natoli, M., 2008. PICK uncoupling from mGluR7a causes absence-like seizures. Nat. Neurosci. 11, 940-8.

Blumenfeld, H., 2005. Cellular and network mechanisms of spike-wave seizures. Epilepsia. 9, 21-33.

Bouwman, B.M., Suffczynski, P., Midzyanovskaya, I.S., Maris, E., van den Broek, P.L., van Rijn, C.M., 2007. The effects of vigabatrin on spike and wave discharges in WAG/Rij rats. Epilepsy Res. 76, 34-40.

Burgess, D.L., Jones, J.M., Meisler, M.H., Noebels, J.L., 1997. Mutation of the Ca2+ channel beta subunit gene Cchb4 is associated with ataxia and seizures in the lethargic (lh) mouse. Cell. 88, 385-92.

Burgess, D.L., Biddlecome, G.H., McDonough, S.I., Diaz ,M.E., Zilinski, C.A., Bean, B.P., Campbell, K.P., Noebels, J.L., 1999. beta subunit reshuffling modifies N- and P/Q-type Ca2+ channel subunit compositions in lethargic mouse brain. Mol Cell Neurosci. 13, 293-311.

Chapman, A.G., Yip, P.K., Yap, J.S., Quinn, L.P., Tang, E., Harris, J.R., Meldrum, B.S., 1999. Anticonvulsant actions of LY 367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid). Eur J Pharmacol. 368, 17-24.

Cheong, E., Zheng, Y., Lee, K., Lee, J., Kim, S., Sanati, M., Lee, S., Kim, Y.S., Shin, H.S., 2009. Deletion of phospholipase C beta4 in thalamocortical relay nucleus leads to absence seizures. Proc Natl Acad Sci U S A. 106, 21912-7.

Coenen, A.M., van Luijtelaar, E.L., 2003. Genetic animal models for absence epilepsy: a review on the WAG/Rij strain of rats. Behav Genet. 33, 635-655.

Conti, V., Aghaie, A., Cilli, M., Martin, N., Caridi, G., Musante, L., Candiano, G., Castagna, M., Fairen, A., Ravazzolo, R., Guenet, J.L., Puliti, A., 2006. crv4, a mouse model for human ataxia associated with kyphoscoliosis caused by an mRNA splicing mutation of the metabotropic glutamate receptor 1 (Grm1). Int J Mol Med. 18, 593-600.

Crunelli, V., Blethyn, K.L., Cope, D.W., Hughes, S.W., Parri, H.R., Turner, J.P., Tòth, T.I., Williams, S.R., 2002. Novel neuronal and astrocytic mechanisms in thalamocortical loop dynamics. Philos Trans R Soc Lond B Biol Sci. 357, 1675-93.

Crunelli, V., Leresche, N., 2002. Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci. 3, 371-82.

Fazio, F., Notartomaso, S., Aronica, E., Storto, M., Battaglia, G., Vieira, E., Gatti, S., Bruno, V., Biagioni, F., Gradini, R., Nicoletti, F., Di Marco, R., 2008. Switch in the expression of mGlu1 and mGlu5 metabotropic glutamate receptors in the cerebellum of mice developing experimental autoimmune encephalomyelitis and in autoptic cerebellar samples from patients with multiple sclerosis. Neuropharmacology. 55, 491-9.

Ferraguti, F., Crepaldi, L., Nicoletti, F., 2008. Metabotropic glutamate 1 receptor: current concepts and perspectives. Pharmacol Rev. 60, 536-81.

Godwin, D.W., Van Horn, S.C., Eriir, A., Sesma, M., Romano, C., Sherman, S.M., 1996. Ultrastructural localization suggests that retinal and cortical inputs access different metabotropic glutamate receptors in the lateral geniculate nucleus. J Neurosci. 16, 8181-92.

Hildebrand, M.E., David, L.S., Hamid, J., Mulatz, K., Garcia, E., Zamponi, GW., Snutch, T.P., 2007. Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11-coupled muscarinic acetylcholine receptors. J Biol Chem. 282, 21043-55.

Hildebrand, M.E., Isope, P., Miyazaki, T., Nakaya, T., Garcia, E., Feltz, A., Schneider, T., Hescheler, J., Kano, M., Sakimura, K., Watanabe, M., Dieudonné, S., Snutch, T.P., 2009. Functional coupling between mGluR1 and Cav3.1 T-type calcium channels contributes to parallel fiber-induced fast calcium signaling within Purkinje cell dendritic spines. J Neurosci. *29*, 9668-82.

Hughes, S.W., Cope, D.W., Blethyn, K.L., Crunelli, V., 2002. Cellular mechanisms of the slow (<1 Hz) oscillation in thalamocortical neurons in vitro. Neuron. 33, 947-58.

Inoue, M., Peeters, B.W., van Luijtelaar, E.L., Vossen, J.M., Coenen, A.M., 1990. Spontaneous occurrence of spike-wave discharges in five inbred strains of rats. Physiol Behav. 48, 199-201.

Izzi, C., Barbon, A., Toliat, M.R., Heils, A., Becker, C., Nürnberg, P., Sander, T., Barlati, S., 2003. Candidate gene analysis of the human metabotropic glutamate receptor type 4 (GRM4) in patients with juvenile myoclonic epilepsy. Am J Med Genet B Neuropsychiatr Genet. 123, 59-63.

Liu, X.B., Muñoz, A., Jones, E.G., 1998. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol. 395, 450-65.

Marignier, R., Chenevier, F., Rogemond, V., Sillevis Smitt, P., Renoux, C., Cavillon, G., Androdias, G., Vukusic, S., Graus, F., Honnorat J, Confavreux C., 2010. Metabotropic glutamate receptor type 1 autoantibody-associated cerebellitis: a primary autoimmune disease? Arch Neurol. 67, 627-30.

Martin, L.J., Blackstone, C.D., Huganir, R.L., Price, D.L., 1992. Cellular localization of a metabotropic glutamate receptor in rat brain. Neuron. 9, 259-70.

Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., Nakanishi, S., 1991. Sequence and expression of a metabotropic glutamate receptor. Nature. 349, 760-765.

McCormick, D.A., Von Krosigk, M., 1992. Corticothalamic activation modulates thalamic firing through glutamate "metabotropic" receptors. Proceedings of the National Academy of Sciences, USA. 89, 2774-2778.

Meeren, H.K., Pijn, J.P., Van Luijtelaar, E.L., Coenen, A.M., Lopes da Silva, F.H., 2002. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. Journal of Neuroscience. 22, 1480-1495.

Miyata, M., Kashiwadani, H., Fukaya, M., Hayashi, T., Wu, D., Suzuki, T., Watanabe, M., Kawakami, Y. 2003. Role of thalamic phospholipase C[beta]4 mediated by metabotropic glutamate receptor type 1 in inflammatory pain. J Neurosci. 23, 8098-108.

Moldrich, R.X., Jeffrey, M., Talebi, A., Beart, P.M., Chapman, A.G., Meldrum, B.S., 2001. Anti-epileptic activity of group II metabotropic glutamate receptor agonists (--)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) and (--)-2-thia-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY389795). Neuropharmacology. 41, 8-18.

Molinaro, G., Traficante, A., Riozzi, B., Di Menna, L., Curto, M, Pallottino, S., Nicoletti, F., Bruno, V., Battaglia, G., 2009. Activation of mGlu2/3 metabotropic glutamate receptors negatively regulates the stimulation of inositol phospholipid hydrolysis mediated by 5-hydroxytryptamine2A serotonin receptors in the frontal cortex of living mice. Mol Pharmacol. 76, 379-87.

Ngomba, R.T., Biagioni, F., Casciato, S., Willems-van Bree, E., Battaglia, G., Bruno, V., Nicoletti, F., van Luijtelaar, E.L., 2005. The preferential mGlu2/3 receptor antagonist, LY341495, reduces the frequency of spike-wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology. 49, 89-103.

Ngomba, R.T., Ferraguti, F., Badura, A., Citraro, R., Santolini, I., Battaglia, G., Bruno, V., De Sarro, G., Simonyi, A., van Luijtelaar, G., Nicoletti, F., 2008. Positive allosteric modulation of metabotropic glutamate 4 (mGlu4) receptors enhances spontaneous and evoked absence seizures. Neuropharmacology. 54, 344-54.

Ngomba, R.T., Santolini, I., Simonyi, A., van Bree, W., Olivieri, G., Molinaro, G., Mairesse, J., Battaglia, G., Bruno, V., van Luijtelaar, G., Nicoletti, F., 2009. Pharmacological activation of metabotropic glutamate receptor subtype 1 dampens spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Soc. Neurosci. Abst. 331.3.

Nicoletti, F., Iadarola, M.J., Wroblewski, J.T., Costa, E., 1986. Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: developmental changes and interaction with α1-adrenoceptors. Proc Natl Acad Sci U S A. 83**,** 1931-1935.

Niswender, C.M., Conn, P.J., 2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol. 50,295-322.

Paxinos, G., Watson, C., 2005. The Rat Brain in the Stereotaxic Coordinates. Academic Press Ltd., London.

Peeters, B.W., van Rijn, C.M., Vossen, J.M., Coenen, A.M., 1989. Effects of GABA-ergic agents on spontaneous non-convulsive epilepsy, EEG and behaviour, in the WAG/Rij inbred strain of rats. Life Sci. 45, 1171-6.

Reichova, I., Sherman, S.M., 2004. Somatosensory corticothalamic projections: distinguishing drivers from modulators. J Neurophysiol. 92, 2185-2197.

Rivadulla, C., Martínez, L.M., Varela, C., Cudeiro, J., 2002. Completing the corticofugal loop: a visual role for the corticogeniculate type 1 metabotropic glutamate receptor. J Neurosci. 22, 2956-62.

Salt, T.E., Turner, J.P., 1998. Reduction of sensory and metabotropic glutamate receptor responses in the thalamus by the novel mGluR1selective antagonist (S) 2-methyl-4-carboxy-phenylglycine. Neuroscience. 85, 655-658.

Schridde, U., van Luijtelaar, G., 2004. The influence of strain and housing on two types of spike-wave discharges in rats. Genes Brain Behav. 3, 1-7.

Shigemoto, R., Nakanishi, S., Mizuno, N., 1992. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J Comp Neurol. 322, 121-35.

Shigemoto, R., Mizuno, N., 2000. Metabotropic glutamate receptors—immunocytochemical and in situ hybridization analyses, in Handbook of Chemical Neuroanatomy, Vol 18, Glutamate (Ottersen OP and Storm-Mathisen J eds), Elsevier Science, New York, pp 63–98.

Sillevis Smitt, P., Kinoshita, A., De Leeuw, B., Moll, W., Coesmans, M., Jaarsma, D., Henzen-Logmans, S., Vecht, C., De Zeeuw, C., Sekiyama, N., Nakanishi, S., Shigemoto, R., 2000. Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor. N Engl J Med. 342, 21-7.

Simonyi, A., Ngomba, R.T., Storto, M., Catania, M.V., Miller, L.A., Youngs, B., DiGiorgi-Gerevini, V., Nicoletti, F., Sun, G.Y., 2005. Expression of groups I and II metabotropic glutamate receptors in the rat brain during aging. Brain Res. 1043, 95-106.

Snead, O.C. 3rd, Banerjee, P.K., Burnham, M., Hampson, D., 2000. Modulation of absence seizures by the GABA(A) receptor: a critical role for metabotropic glutamate receptor 4 (mGluR4). J Neurosci. 20, 6218-24.

Stinehelfer, S., Vruwink, M., Burette, A., 2000. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 861, 37-44.

Turner, J.P., Salt, T.E., 2000. Synaptic activation of the Group I metabotropic glutamate receptor mGlu1 on the thalamocortical neurones of the rat dorsal lateral geniculate nucleus *in vitro*. Neuroscience. 100, 493-505.

van Luijtelaar, E.L., Coenen, A.M., 1986. Two types of electrocortical paroxysms in an inbred strain of rats. Neuroscience Letters. 70, 393-397.

van Luijtelaar, E.L., Coenen, A.M., 1988. Circadian rhythmicity in absence epilepsy in rats. Epilepsy Res. 2, 331-6.

van Luijtelaar, G., Sitnikova, E., 2006. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 30, 983-1003.

van Rijn, C.M., Gaetani, S., Santolini, I., Badura, A., Gabova, A., Fu, J., Watanabe, M., Cuomo, V., van Luijtelaar, G., Nicoletti, F., Ngomba, R.T., 2010. WAG/Rij rats show a reduced expression of CB receptors in thalamic nuclei and respond to the CB receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. Epilepsia. 51, 1511-21

Vidnyanszky, Z., Gorcs, T.J., Negyessy, L., Borostyankio, Z., Knopfel, T., Hamori, J., 1996. Immunocytochemical visualization of the mGluR1a metabotropic glutamate receptor at synapses of corticothalamic terminals originating from area 17 of the rat. Eur J Neurosci. 8, 1061-71.

Vieira, E., Huwyler, J., Jolidon, S., Knoflach, F., Mutel, V., Wichmann, J., 2009. Fluorinated 9H-xanthene-9-carboxylic acid oxazol-2-yl-amides as potent, orally available mGlu1 receptor enhancers. Bioorg Med Chem Lett. 19, 1666-9.

Wang, X., Ai, J., Hampson, D.R., Snead, O.C. 3rd, 2005. Altered glutamate and GABA release within thalamocortical circuitry in metabotropic glutamate receptor 4 knockout mice. Neuroscience. 134, 1195-203.

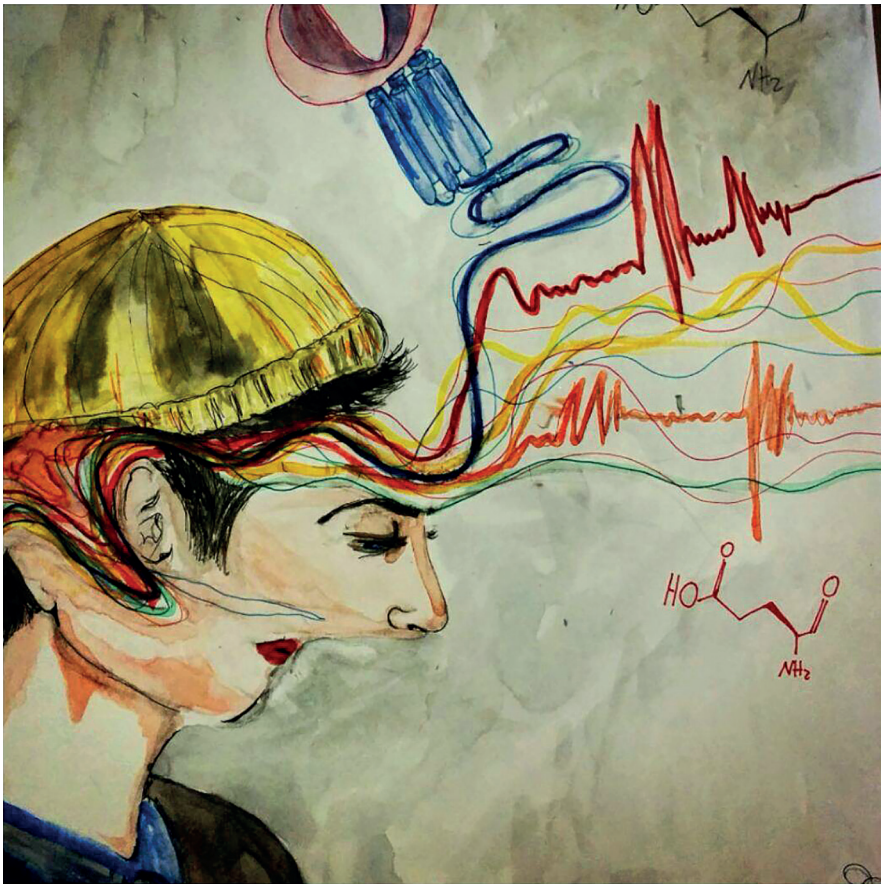
Watanabe, M., Nakamura, M., Sato, K., Kano, M., Simon, M.I., Inoue, Y., 1998. Patterns of expression for the mRNA corresponding to the four isoforms of phospholipase Cbeta in mouse brain. Eur J Neurosci. 10, 2016-25.

Wong, C.G., Scherer, S.W., Snead, O.C. 3rd, Hampson, D.R., 2001. Localization of the human mGluR4 gene within an epilepsy susceptibility locus(1). Brain Res Mol Brain Res. 87, 109-16.

Yu, L., Blumenfeld, H., 2009. Theories of impaired consciousness in epilepsy. Ann N Y Acad Sci. 1157, 48-60.

Zhang, L., Jones, E.G., 2004. Corticothalamic inhibition in the thalamic reticular nucleus. J Neurophysiol. 91, 759-66.

Zhang, C.S., Bertaso, F., Eulenburg, V., Lerner-Natoli, M., Herin, G.A., Bauer, L., Bockaert, J., Fagni, L., Betz, H., Scheschonka, A., 2008. Knock-in mice lacking the PDZ-ligand motif of mGluR7a show impaired PKC-dependent autoinhibition of glutamate release, spatial working memory deficits, and increased susceptibility to pentylenetetrazol. J Neurosci. 28, 8604-14.




# 3 Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats

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## Abstract

Absence epilepsy is generated by the cortico-thalamo-cortical network, which undergoes a finely tuned regulation by metabotropic glutamate (mGlu) receptors. We have shown previously that activation of mGlu1 receptors reduces spontaneous spike and wave discharges (SWDs) in the WAG/Rij rat model of absence epilepsy, whereas activation of mGlu2/3 and mGlu4 receptors produces the opposite effect. Here, we have extended the study to mGlu5 receptors, which are known to be strongly expressed within the cortico-talamo-cortical network. WAG/Rij rats showed a reduction in the mGlu5 receptor protein levels and in the mGlu5-receptor mediated stimulation of polyphosphoinositide hydrolysis in the ventrobasal thalamus, whereas the expression of mGlu5 receptors was increased in the somatosensory cortex. Interestingly, these changes preceded the onset of the epileptic phenotype, being already visible in pre-symptomatic WAG/Rij rats. Absence seizures in 8-month old WAG/Rij rats were not influenced by pharmacological blockade of mGlu5 receptors with MTEP (10 or 30 mg/kg, i.p.), but were drastically decreased by mGlu5 receptor activation with the novel enhancer, VU0360172-6 (3 or 10 mg/kg, i.p.). The action of VU0360172-6 was prevented by co-treatment with MTEP. These findings suggest that changes in mGlu5 receptors might lie at the core of the absence-seizure prone phenotype of WAG/Rij rats, and that mGlu5 receptor enhancers are potential candidates to the treatment of absence epilepsy.


**Keywords**: Absence epilepsy, WAG/Rij rats, mGlu5 receptor, VU0360172-6

# 1. Introduction

Typical absence epilepsy is a generalized non convulsive form of epilepsy characterized by bilaterally synchronous spike and wave discharges (SWDs) that spreads all-over the cortex as seen on the electroencephalogram (reviewed by Blumenfeld, 2005). SWDs are generated within a cortico-thalamo-cortical loop that comprises cortical pyramidal neurons and GABAergic interneurons, thalamic relay neurons, and inhibitory neurons of the reticular thalamic nucleus (nRT) and neurons of the nRT are also connected to each other via inhibitory GABAergic synapses as well as by gap junctions (reviewed by Blumenfeld, 2005; Meeren et al., 2002; Polack et al., 2009). The cortex (layer V and VI) send excitatory glutamatergic projections to the nRT and thalamic relay neurons send excitatory glutamatergic projections to cortical pyramidal neurons and nRT. The nRT neurons send inhibitory GABAergic connections to thalamic relay neurons but not to the cortex (Zhang and Jones, 2004; Blumenfeld, 2005; van Luijtelaar and Sitnikova, 2006). The nature of SWDs generated within the cortico-thalamo-cortical network has led to several debates on which anatomical area might be held as an initiation site and pioneer studies have tentatively put together the importance of the thalamus (Jasper and Kershman, 1941) and the cerebral cortex (Avoli and Gloor, 1982) as key players. These studies have demonstrated that there is an aberrant function within the cortico-thalamo-cortical loop that contributes to the pathophysiology of absence seizure. In this loop the metabotropic glutamate (mGlu) receptors are particularly positioned to modulate rather than mediate synaptic transmission and are therefore potential targets for the management of SWDs (reviewed by Ngomba et al., 2011a). Group-I mGlu receptors (mGlu1 and mGlu5 receptors) are coupled to $Gq/G_{11}$ proteins and are predominantly localized in the peripheral portion of postsynaptic densities. Their activation stimulates polyphosphoinositide hydrolysis with formation of inositol-1,4,5-trisphosphate and diacylglycerol, and also regulate the activity of different types of calcium and potassium channels (reviewed by Nicoletti et al., 2011). mGlu1 and mGlu5 receptors show a different pattern of distribution in the cortico-thalamo-cortical network, which suggests distinct rather than complementary functions of these two receptor subtypes. High densities of mGlu1 receptors are found on dendrites of thalamic relay neurons that receive afferent input from cortical layer VI neurons (Ferraguti et al., 2008; reviewed by Ngomba et al., 2011a). In the cortex, mGlu1 receptors appear to be exclusively expressed postsynaptically on GABAergic interneurons (Stinehelfer et al., 2000). Moreover, it has been shown that activation of mGlu5 receptors has an excitatory effect similar to that mediated by mGlu1 receptors and that these receptors can participate in sensory responses of thalamic relay cells (Salt and Binns, 2000). Thalamic relay neurons also expressed mGlu5 receptors at dendrites postsynaptic to cortical inputs (Romano et al., 1995; Liu et al., 1998). Neurons of the nRT also show moderate-to-low expression of mGlu5 receptors (Romano et al., 1995; Lourenço Neto et al., 2000). In the cerebral cortex, mGlu5 receptors are expressed by pyramidal neurons

postsynaptic to thalamo-cortical projections (Wijetunge et al., 2008), as well as by interneurons (Romano et al., 1995; Sun et al., 2009). We have recently examined the role of mGlu1 receptors in absence seizures using Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats as a genetic model of absence epilepsy. WAG/Rij rats develop spontaneous SWDs at >2 months of age, and at 5-6 months of age they all have hundreds of SWDs per day (Coenen and van Luijtelaar, 1987; Schridde and van Luijtelaar, 2004). In these rats, generalized bilateral SWDs are characterized by a frequency of 7-9 Hz and duration of 1-30 sec, and are associated with behavioural manifestation mimicking those of absences in humans (Coenen and van Luijtelaar, 2003; van Luijtelaar and Sitnikova, 2006). SWDs in WAG/Rij rats are reduced by classical anti-absence drugs, such as ethosuximde and valproate, and are increased by vigabatrin and carbamazapine, which worsen absence seizures in humans (Bouwman et al., 2007; Coenen and van Luijtelaar, 2003; Peeters et al., 1989). Thus, WAG/Rij rats represent a model of absence epilepsy endowed with construct, face, and pharmacological validity. We have reported that mGlu1 receptors are down-regulated in the ventral basal thalamus of symptomatic WAG/Rij rats, and that pharmacological activation of mGlu1 receptors reduces SWDs in these rats (Ngomba et al., 2011b). We have now extended the study to mGlu5 receptors, finding that receptor expression and function change in the thalamus and somatosensory cortex of both pre-symptomatic and symptomatic WAG/Rij rats, and that a selective mGlu5 receptor enhancer drastically reduce absence seizures.

# 2. Materials and methods

## 2.1. Drugs

VU0360172-6 (N-cyclobutyl-6-[2-(3-fluorophenyl)ethynyl]pyridine-3-carboxamide) was obtained from Vanderbilt University Medical Center, JNJ16259685 (( 3,4-dihydro-2H-pyranol [2,3-b] quinolin-7yl) (cis-4-ethoxycycloexyl)methanone),DHPG (3,5 dihydroxyphenylglycine), MTEP3-((2-Methyl-1,3-thiazol-4-yl) ethynyl) pyridine hydrochloride, and diazepam were purchased from Tocris Cookson Ltd. (Bristol, UK); VU0360172-6 was dissolved in 10% Tween 80, and injected s.c.. MTEP was dissolved in saline, and injected i.p..

## 2.2. Animals

Male WAG/Rij and ACI (Agouti Copenhagen Irish) rats were kept under environmentally controlled conditions (ambient temperature = 22 °C, humidity = 40%) in a room with reversed light-dark cycle (light on from 8:00 p.m. to 8:00 a.m.), with food and water *ad libitum*. Experiments were carried out during the dark phase of the cycle in which WAG/Rij rats have the largest amount of SWDs (van Luijtelaar and Coenen, 1988). All animals were handled prior to EEG registrations. For biochemical studies, age-matched ACI rats were used as controls. ACI rats have no or only very few SWDs and the lowest number of SWDs of all inbred strains investigated as was assessed in a 48 h EEG evaluation study (Inoue et

al., 1990) and in all cases they have much less SWDs than WAG/Rij rats of the same age (Schridde and van Luijtelaar, 2004). Therefore, ACI rats are commonly used as controls in experiments with WAG/Rij rats (Lasoń et al., 1992; Ngomba et al., 2008; van de Bovenkamp-Janssen et al., 2006; van Rijn et al., 2010). We used rats of both strains at 2 and 8 months of age. WAG/Rij rats of 2 months of age do not show SWDs as yet, and, therefore, are considered "pre-symptomatic". All 8 months old WAG/Rij rats have about 16–20 SWDs per hour, which is a few hundred SWDs per day, and are thus defined as "symptomatic". This study was performed in accordance with the guidelines of the European Community for the use of experimental animals and approved by the local ethics committee for animal studies (RU-DEC).

## 2.3 Immunoistochemisrty

Brains from WAG/Rij and ACI rats were fixed in Carnoy's solution (60% ethanol, 10% acetic acid, and 30% chloroform), embedded in paraffin and cut into sections of 10 mm. Deparaffinised sections were pre-incubated for 1 h in normal goat serum for 1 h (vector laboratories, Burlingame, CA), then incubated overnight with polyclonal rabbit anti-mGluR5 (1:100; Millipore, Billerica, MA) antibody dissolved in 10% of PBS and then for 1 h with secondary biotin-coupled anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA). 3,3-Diaminobenzidine tetrachloride (Sigma Aldrich, Milan, Italy) was used for detection. Control staining was carried out without the primary antibody. The intensity of mGlu5 immunoreactivity in cortical and thalamic regions (see Fig.1) was quantified by measuring the relative optical densities. Brain coronal sections of 350 mm were sliced from bregma -1.3 mm to -4.8 mm. Images were acquired at low magnification (2.5x) and the densitometric anal- ysis was performed by assessing the intensity of the background values (i.e., the optical density measured in unlabelled areas present in the section, such as corpus callosum) by using Zeiss Axio Imager M1 microscope equipped with a motorized stage and focus control system (Zeta axis), and with a digital video camera.

## 2.4. Western blot analysis of mGlu5 receptors

Male WAG/Rij and matched control ACI rats, 2 or 8 month old, were anesthetized with ether, decapitated and brains were rapidly removed and frozen. Brains were coded and the codes were released after Western blot analysis. Whole brains were placed on a cryostat and tissue that comprises the principal area of the cortico-thalamo-cortical network were dissected out using the coronal diagrams (between coordinates from bregma -1.88 mm and - 3.80 mm) from Paxinos and Watson atlas (2005) as a guide. For cortical areas, we dissected out a part of the cortex that includes primary somatosensory cortex (S1) and another portion of the cortex that includes primary motor cortex (M1). Then, the thalamus was dissected into two separate parts, one containing the reticular thalamic nucleus (nRT) and the second containing all other thalamic nuclei, as previously described (Ngomba et al., 2008, 2011b). Cortical areas 2 mm anterior and posterior from

the above mentioned cortical areas were dissected out and used as control regions. Moreover, the hippocampus, which is thought not to be involved in the pathogenesis of absence seizures was also dissected. The expression of mGlu5 receptor proteins was estimated by Western blot analysis, using a specific rabbit polyclonal antibody (1:1.000, Upstate Biotechnology, Lake Placid, NY) and a mouse monoclonal antibody to label β-actin (1:100.000, Sigma, St.Louis, MO). Brain tissues were homogenized at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin, and 1 mg/ml leupeptin. Proteins were resuspended in SDS-bromophenol blue reducing buffer with 40 mM DTT. Western blot analyses were carried out using 8% SDS polyacrylamide gels which were electroblotted on immunblot PVDF membranes (BioRad, Milano, Italy); filters were blocked overnight in TBS-T buffer containing 5% non fatty milk. Blots were incubated for 1 h with rabbit polyclonal anti-mGlu5 antibodies and mouse monoclonal antibody to label β-actin. Filters were washed with TBS-T buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit or anti-mouse, Amersham, Piscataway, NJ) diluted 1:7000 with TBS-T buffer. Immunostaining was revealed by enhanced ECL.

## 2.5. Measurement of DHPG-stimulated polyphosphoinositide (PI) hydrolysis in living rats

ACI and WAG/Rij rats were anesthetized with ketamine (100 mg/kg) plus xylazine (10 mg/kg) and injected with [*myo*-$^{3}$H]inositol (2 µCi/5 µl/2 min, i.c.v.). Twenty-four hours later, rats were treated with lithium ions (administered as LiCl, 10 mmol/kg, s.c.) to inhibit the conversion of inositol monophosphate (InsP) into free inositol. The mGlu1/5 receptor agonist, DHPG (500 nmol/5 µl saline containing 50% dimethyl sulfoxide) was injected i.c.v., 1 h after LiCl injection. Control rats were injected with the vehicle alone. The selective mGlu1 receptor antagonist JNJ16259685 (10 mg/kg) and diazepam (10 mg/kg) used as an anti-convulsant drug, were injected i.p. 30 min before DHPG. Rats were killed 1 h after treatment with DHPG or vehicle. The sensorymotor cortex and the thalamus were quickly removed and stored at −80 °C. On the day of the assay, tissue was sonicated in 1.25 ml of water containing 10 mM LiCl. After centrifugation at 10,000 *g* for 20 min, the [$^{3}$H] InsP present in the supernatant was separated by anion exchange chromatography in 10 ml columns containing 1.5 ml of Dowex 1-X-8 resin (formate form, 100–200 mesh; Bio-Rad). Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP (Nicoletti et al., 1986). Total radioactivity in the brain regions was determined by counting a 100 µl aliquot of the whole homogenate.

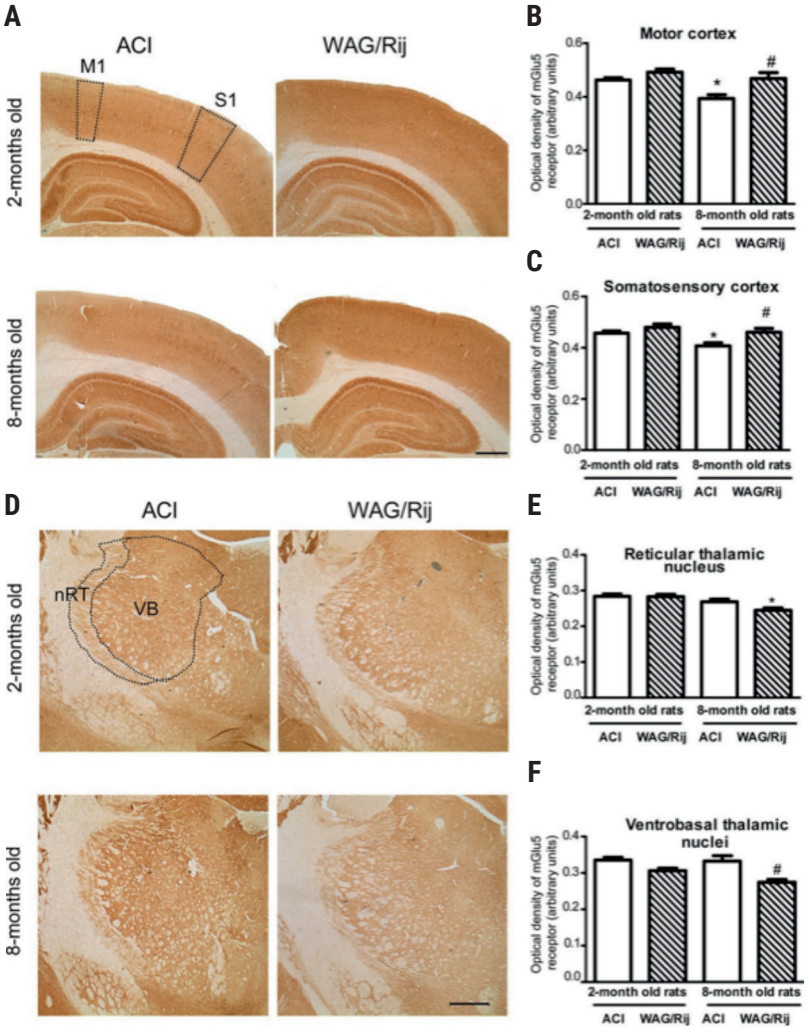


**Figure 1** Immunohistochemical analysis of mGlu5 receptors in the cortico-thalamic-cortical network of ACI and WAG/Rij rats. Representative mGlu5 receptor immunostaining in the cortex (M1 and S1) and thalamus (nRT and VB) of ACI and WAG/Rij rats at 2 and 8 months of age are shown in (A) and (D), respectively. The densitometric analysis was performed in M1, S1, nRT and VB (the areas are delineated with black discontinuous illustrative boxes in A, D). Data of densitometric analysis are shown in (B, C, E, F). Values are means ± S.E.M. and were calculated from the average of 5 coronal sections for each animal (n = 6 animals per group). *$p < 0.05$ vs the corresponding values obtained in the same strain at 2 months, #$p < 0.05$ vs. the corresponding values obtained in ACI rats at 8 months.

## 2.6. EEG recordings

Male WAG/Rij rats (8-month old) with a mean body weight of 390 ± 20 g, were used. Animals were individually housed in Macrolon cages in a room illuminated with white lights from 8:00 p.m. to 8:00 a.m. A permanent cortical tripolar electrode set was implanted under complete isoflurane anesthesia, one electrode into the frontal region (coordinates with the skull surface flat and from bregma zero–zero, AP +2.0; L –2.5), and the other in the parietal region (A –6.0; L –4.0) (Paxinos and Watson, 2005). The ground electrode was placed over the cerebellum. After the surgery, animals were allowed to recover for two weeks. Rats were put into transparent recording cages, connected to an EEG cable which allowed free movement, and habituated to the experimental conditions for 12 h. The EEG was filtered (only frequencies between 0.1 and 100 Hz, were allowed to pass, digitalized with a sample frequency of 512 Hz, and stored for off-line analysis using Windaq system (DATAQ Instruments, Akron, OH, USA). Baseline EEG was recorded during the dark period (9:00–11:00 a.m.) for 2 h prior to injection and thereafter, following the systemic injection of the selective mGlu5 receptor positive allosteric modulator, VU0360172-6 (3 or 10 mg/kg, s.c., dissolved in tween) and the mGlu5 receptor antagonist, MTEP (10 or 30 mg/kg, i.p., dissolved in saline), or vehicle in a volume of 1 ml. The post injection EEG was registered during the next 5 h. SWDs were marked at visual inspection based on commonly used criteria: trains of sharp spikes and slow waves lasting minimally 1 s, an amplitude of the spikes at least twice the background, frequency of the SWDs between 7 and 10 Hz and an asymmetric appearance of the SWDs (van Luijtelaar and Coenen, 1986).

## 2.7. Animal behaviour: recording of spontaneous motor activity

Spontaneous motor activity in symptomatic WAG/Rij rats was recorded with an analogic passive infrared detector (PIR) (Luna PR, Rokonet Electronics LTD, Rishon Le Tzion, Israel) (van Rijn et al., 2010) during the EEG recording session. The analogue signal was digitalized simultaneously with the EEG signal. In order to check whether the drugs induced behavioural effects and whether the putative drug effects on SWDs could be ascribed to effects on behaviour considering the initmate relation- ship between the behavioural state of the animal and the occurrence of SWDs (Drinkenburg et al., 1991; Smyk et al., 2011), the amount of bodily movements in the baseline and after drug administration were quantified by calculating the mean of the PIR signal per hour.

## 2.8. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis for Western blot and immunohistochemistry was carried out by a two-way ANOVA, with the rat strain and age as independent factors, while Student's t-test was used for the analysis of DHPG-stimulated PI hydrolysis. Statistical analysis of EEG and behavioural data was carried out by ANOVA for repeated measures followed by the Bonferroni's test to isolate the differences.

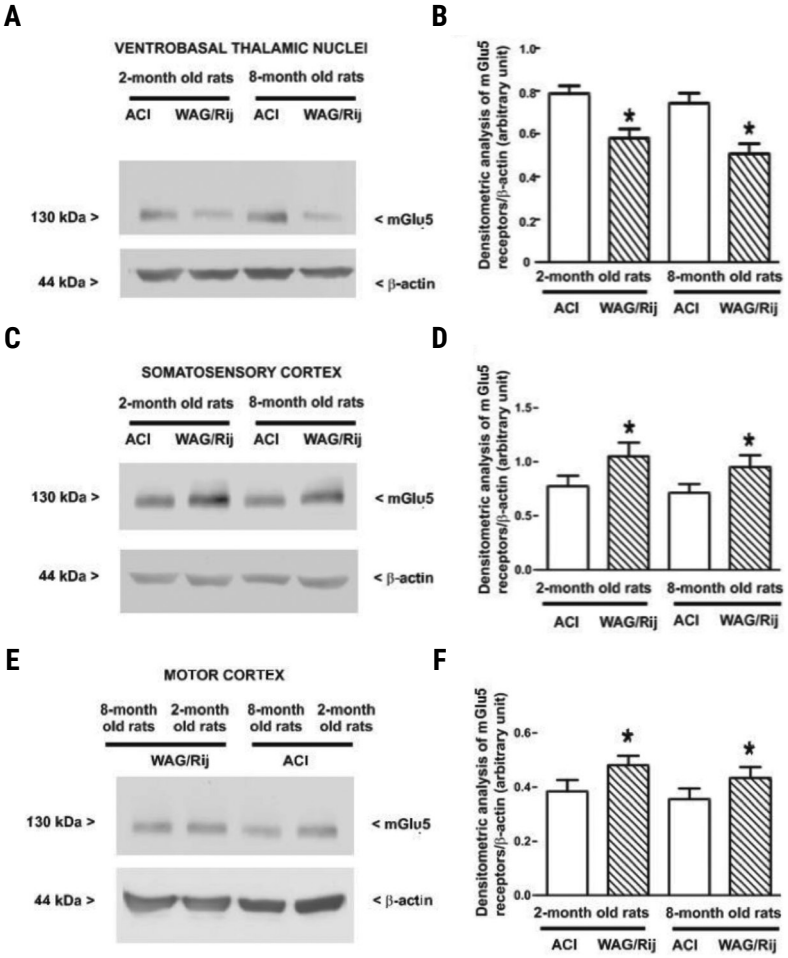


**Figure 2** Immunoblot analysis of mGlu5 receptors in the ventrobasal thalamus and sensorymotor cortex of ACI and WAG/Rij rats. Representative immunoblot of mGlu5 receptors in the ventrobasal thalamic nuclei (A), somatosensory cortex (C) and motor cortex (E) of 2- and 8-month old ACI or WAG/Rij rats are shown. Densitometric analysis is shown in (B), (D), and (F), respectively. Statistical analysis was carried out by two-way ANOVA, with the rat strain and age as variable factors. Values are means ± S.E.M. (n = 8 animals per group). Post hoc analysis: *$p < 0.05$ vs. age-matched control ACI rats. In each individual blot, protein extracts from rats of each experimental group were loaded in order to obtain relative comparisons among the groups.

# 3. Results

## 3.1. Expression of mGlu5 receptors in the cortico-thalamo-cortical network of ACI and WAG/Rij rats at 2 and 8 months of age

Immunohistochemical analysis of mGlu5 receptors in the sen- sorymotor cortex and thalamus of ACI and WAG/Rij rats at 2 and 8 months of age is shown in Fig. 1A and D, respectively. In the motor and somatosensory cortices of non-epileptic ACI rats, expression of mGlu5 receptors decreased with age, being lower at 8 than at 2 months of age. In contrast, expression of mGlu5 receptors in the motor and somatosensory cortices of WAG/Rij rats did not change with age. Eight-month old symptomatic WAG/Rij rats showed a significant increase in mGlu5 receptor immunoreactivity in the motor and somatosensory cortices as compared to age-matched ACI rats (Fig. 1B and C). A trend to an increased immuno-reactivity was also seen in 2-month old presynthomatic WAG/Rij rats with respect to age-matched ACI rats. However, this difference did not reach statistical significance (Fig. 1B and C). In contrast, 8-month old WAG/Rij rats showed a reduction in mGlu5 receptor immuno- reactivity in both the nRT and ventrobasal thalamic nuclei (VB). In the nRT, mGlu5 receptor immunoreactivity was significantly lower in 8-month old WAG/Rij rats than in 2-month old WAG/Rij rats (Fig. 1E), In the VB, a significant reduction was found between 8- month old WAG/Rij rats and age-matched ACI rats (Fig. 1F). A nonsignificant trend to a reduction in mGlu5 receptor immunore- activity was also seen in the VB of 2-month old WAG/Rij rats, as compared to ACI rats of corresponding age (Fig. 1F).

We also carried out immunoblot analysis of mGlu5 receptors on protein extracts from the motor cortex, somatosensory cortex, and VB of ACI and WAG/Rij rats. Immuno-blots showed a major band at about 130 kDa corresponding to the mGlu5 receptor monomers. Data of Western blot analysis were in line with those obtained by immuno-histochemistry showing an increased mGlu5 receptor expression in the motor and somatosensory cortices, and a reduced expression in the VB of 8-month old symptomatic WAG/Rij rats as compared to age-matched ACI rats (Fig. 2AeF). The only difference was that these changes reached statistical significance also in 2- month old WAG/Rij rats (vs. age-matched ACI rats) (Fig. 2AeF). Thus, at least immunoblot data suggest that changes of mGlu5 receptors in the cortico-thalamic-cortical network of WAG/Rij rats precede the onset of SWDs.

## 3.2. Changes in mGlu5 receptor-mediated PI hydrolysis in the cortex and thalamus of WAG/Rij rats

We used an in vivo metho to assess the mGlu5-receptor mediated PI hydrolysis in the sensorymotor cortex and thalamus of ACI and WAG/Rij rats (see Fig. 3A for experimental details). Rats were infused i.c.v. with the mixed mGlu1/5 receptor agonist, DHPG (500 nmol/5 ml), and the component of PI hydrolysis mediated by mGlu5 receptors was isolated by co-injecting the rats with the mGlu1 receptor antagonist, JNJ16259685 (10 mg/kg, i.p.).

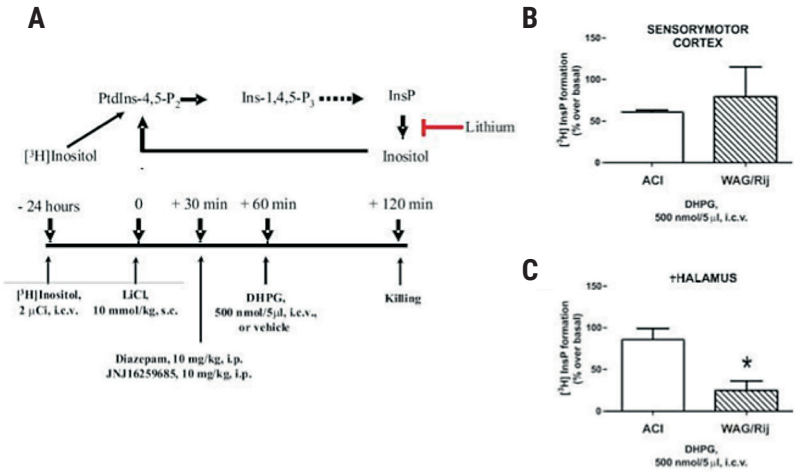


**Figure 3** Changes in mGlu5 receptor-mediated PI hydrolysis in the sensorymotor cortex and thalamus of WAG/Rij rats. In vivo measurements of PI hydrolysis in the sensorymotor cortex and thalamus of 8-month old WAG/Rij rats and age-matched controls (ACI rats) was carried out as outlined in (A). PtdIns-4,5,P2 = phosphatidylinositol-4,5-bisphosphate; Ins-1,4,5-P3 = inositol-1,4,5-trisphosphate; InsP = inositolmonophosphate. DHPG-stimulated PI hydrolysis in the sensorymotor cortex (B) and in thalamus (C) of WAG/Rij rats (n = 5 animals per group). *p < 0.05 (Student's t test) vs. values obtained in controls rats. Basal values of [3H] InsP formation normalized by the amount of total radioactivity in each sample were 1.45 ± 0.17 and 1.40 ± 0.15 (means ± S.E.M.) in the sensorymotor cortex of ACI and WAG/Rij rats, respectively. Basal values of [3H] InsP formation normalized by the amount of total radioactivity in each sample were 1.68 ± 0.08 and 1.72 ± 0.12 (means ± S.E.M.) in the thalamus of ACI and WAG/Rij rats, respectively.

There was no significant change in DHPG-stimulated PI hydro- lysis in the sensorymotor cortex between WAG/Rij and ACI rats, although a trend to an increase was seen in WAG/Rij rats (Fig. 3B). In contrast, a large reduction in DHPG-stimulated PI hydrolysis was seen in the thalamus of WAG/Rij rats (Fig. 3C), in close agreement with receptor expression data.

## 3.3. Pharmacological potentiation of mGlu5 receptors reduced spike and wave discharges (SWDs) in WAG/Rij rats

Acute treatment with MTEP (10 or 30 mg/kg, i.p.), a selective negative allosteric modulator (NAM) of mGlu5 receptors (Anderson et al., 2002; Cosford et al., 2003), did not change the incidence and mean duration of SWDs in symptomatic WAG/Rij rats (Fig. 4A and B). We next examined whether pharmacological potentiation of mGlu5 receptors could affect SWDs by injecting rats with the mGlu5 receptor enhancer, VU0360172. This drug was used

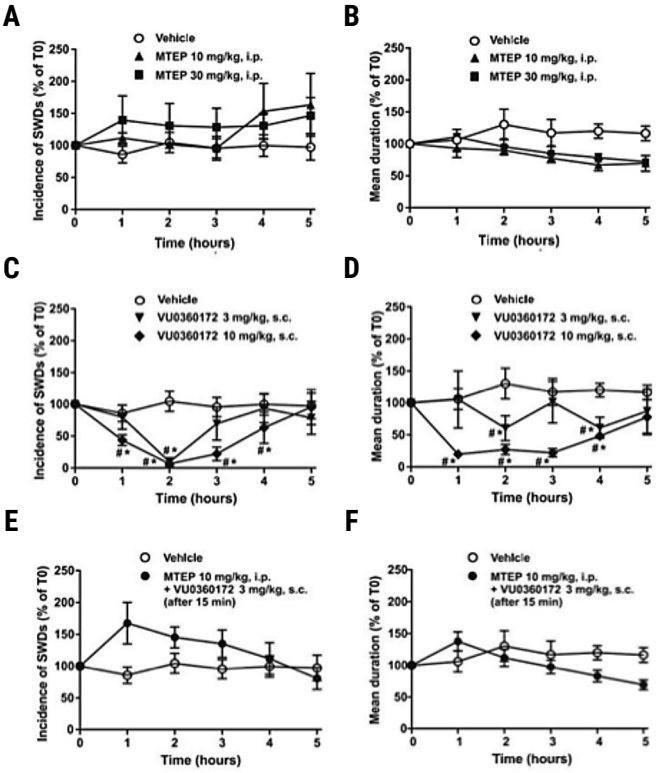


**Figure 4** Pharmacological potentiation of mGlu5 receptors reduces spike and wave discharges (SWDs) in WAG/Rij rats. Pharmacological potentiation of mGlu5 receptors reduces both number and mean duration of SWDs in symptomatic WAG/Rij rats (C, D). No effect of MTEP alone (A, B) or combined with VU0360172 (E, F) on number and mean duration of SWDs. Values are means ± S.E.M. of 4/8 animals (MTEP 10 mg/kg = 4 rats, MTEP 30 mg/kg = 6 rats, VU0360172 3 or 10 mg/kg = 6 rats, MTEP þ VU0360172 = 8 rats). $p < 0.05$ (ANOVA for repeated measures followed by Bonferroni's test) vs. the corresponding values at baseline (T0) (*) or vs. the corresponding values obtained in WAG/Rij rats treated with vehicle (#).

at doses (3 or 10 mg/kg, s.c.) that are known to be centrally active (Rodriguez et al., 2010). Pharmacological enhancement of mGlu5 receptors signifi- cantly reduced the incidence of SWDs in a dose-dependent manner in the first 2 h after administration, and the effect of the highest dose lasted up to 4 h after drug injection (Fig. 4C). Treatment with VU0360172 also reduced the mean duration of SWDs to a similar extent as the incidence of SWDs (Fig. 4D). To exclude unknown off- target effects of VU0360172, the compound

was also injected at the dose of 3 mg/kg, s.c., in WAG/Rij rats pretreated with MTEP (10 mg/ kg, i.p.) 15 min earlier. As shown in Fig. 4E and F, the effect of VU0360172 was antagonized by MTEP, demonstrating that the drug reduced absence seizures by amplifying the endogenous activation of mGlu5 receptors. Spectral analysis of the EEG during SWDs after the highest dose of VU0360172 showed that the peak frequency of the SWDs was7.9 Hz, and did not differ from the peak frequency detected in WAG/ Rij rats treated with vehicle (data not shown).

### 3.4. Pharmacological potentiation of mGlu5 receptors did not affect motor behaviour

To exclude that changes in SWD induced by VU0360172 were secondary to changes in motor behaviour, we measured motor activity in 8-month old WAG/rij rats for 7 h following the injection of vehicle or VU0360172 (3 or 10 mg/kg, s.c.). ANOVA for repeated measures showed a significant effect of time ($F = 5.77$, df 5,14, $p < 0.001$), with no drug effect and no drug x time interaction (Fig. 5).



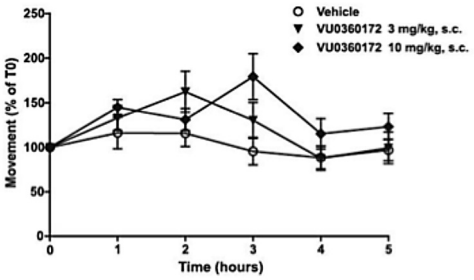


**Figure 5** Assessment of motor behaviour in 8-month WAG/Rij rats treated with vehicle or VU0360172. Values are means þ S.E.M. (n = 6 rats per group). ANOVA for repeated measures showed a significant effect of time with no drug effect and no drug x time interaction.

## 4. Discussion

A major finding here was that pharmacological enhancement of mGlu5 receptor activity caused a robust and dose-dependent reduction in the incidence and mean duration of SWDs in WAG/Rij rats, which have pharmacological validity as a model for absence epilepsy. In contrast, pharmacological blockade of mGlu5 receptors did not affect absence seizures. A different scenario occurs in models of convulsive seizures, where pharmacological blockade of mGlu5 receptors is usually protective (Chapman et al., 2000; Lojková and Mares, 2005; Jesseet al., 2008). WAG/Rij rats also showed a reduced expression and

function of mGlu5 receptors in the thalamus and an increased receptor expression in the sensor- ymotor cortex. Immunoblot data show that in WAG/Rij rats changes in mGlu5 receptor expression are antecedent to the onset of absence seizures, and, therefore, are not secondary to seizure activity. This is peculiar because changes in the expression of mGlu1 receptors in the thalamus only occur in symptomatic WAG/ Rij rats (Ngomba et al., 2011b), whereas expression ofmGlu4 receptors show opposite modifications in pre-symptomatic and symptomatic WAG/Rij rats (Ngomba et al., 2008). We wish to highlight that the reduced function of mGlu5 receptors in the thalamus was seen using an in vivo method that allows the assessment of agonist-stimulated PI hydrolysis without the biases inherent to the conventional assay of PI hydrolysis in brain slices. We suggest that a reduced expression and function of mGlu5 receptors in the thalamus is a molecular determinant of the pathological phenotype of WAG/Rij rats, and that pharmacological amplification of thalamic receptors that are still present and func- tional is sufficient to significantly reduce absence seizures. If so, it is not surprising that MTEP had no effect on SWDs because thalamic mGlu5 receptors were already hypofunctional in WAG/Rij rats. It is worth mentioning that another mGlu5 receptor NAM, 2-methyl-6-(phenylethynyl) pyridine (MPEP) inhibits SWDs in lethargic mice (Chapman et al., 2000), which represent an atypical model of absence epilepsy and bear a mutation in the b4 subunit of voltage-sensitive calcium channels (Burgess et al., 1999). However, MPEP is also a weak mGlu4 receptor PAM (Mathiesen et al., 2003) and mGlu4 receptors are involved in the generation of absence seizures (Snead et al., 2000; Ngomba et al., 2008). MPEP had no effect on acutely induced SWDs by low doses of the GABAA receptor antagonist, pentylenetetrazol, in immature rats (Lojková and Mares, 2005). The logical conclusion is that the role of mGlu5 receptors in different seizure and epilepsy models is not uniform, and the effect of mGlu5 receptor NAMs critically depends on the genetic background of the animals, the age and/or the type of absence seizures (e.g., spontaneous vs. drug-induced). The mGlu5 receptor is physically linked to the NR2 subunit of N-methyl-D-aspartate (NMDA) receptors through a chain of scaf- folding proteins that include PSD95, Shunk, and Homer (Tu et al., 1999). Furthermore, activation of mGlu5 receptors facilitates the activation of NMDA receptors, and vice versa (Doherty et al., 1997; Ugolini et al., 1999; Awad et al., 2000; Attucci et al., 2001; Mannaioni et al., 2001; Pisani et al., 2001; Alagarsamy et al., 2005). This interaction cannot explain the protective effect of VU0360172 that we have observed here because it is the NMDA receptor blockade that reduces absence seizures in WAG/Rij rats (Peeters et al. 1990; Coenen and van Luijtelaar, 2003). Either neuronal mGlu5 receptors act independently of the NMDA receptors in regulating synaptic activity in the cortico-thalamo-cortical network, or glial mGlu5 receptors (Liu et al., 1998) have a role in regulating the expression of high affinity glutamate transporters (Aronica et al., 2003) or signalling molecules that may affect the function of thalamic or cortical neurons. An attractive hypothesis is that activation of mGlu5 receptors in VB restrains GABA release and therefore protects against SWDs through a mechanism mediated by an enhanced formation of en-

docannabinoids with ensuing activation of presynaptic CB1 cannabinoid receptors (Varma et al., 2001; Jung et al., 2005; Uchigashima et al., 2007; Won et al., 2009; Izumi and Zorumski, 2012). Interestingly, pharmacological activation of CB1 receptors reduces absence seizures in WAG/Rij rats (van Rijn et al., 2010). It is worth noting that systemic injection of drugs that enhance synaptic GABA levels, such as tiagabine and vigabatrin, enhance SWDs in GAERS and WAG/Rij rats (Coenen et al., 1995; Depaulis and van Luijtelaar, 2006; Bouwman et al., 2007), and similar effects are seen following bilateral injections of g-vinyl GABA or the GABAA receptor agonist, muscimol, into the medial part of the ventral lateral thalamus containing thalamic relay nuclei (Liu et al., 1991). In contrast, injections of g-vinyl GABA or muscimol into the nRT suppress SWDs (Liu et al., 1991). Local injections with VU0360172 in the VB or nRT in combination with CB1 receptor antagonists and/or GABAergic drugs may help to clarify the precise mechanism whereby pharmacological enhancement of mGlu5 receptors reduces absence seizures in WAG/Rij rats. We have also found an increased expression of mGlu5 recep- tors in the sensorymotor cortex of WAG/Rij rats, although receptor function was not significantly enhanced. The significance of this finding is uncertain because pharmacological blockade of mGlu5receptors did not affect absence seizures.

We cannot exclude that an enhanced expression of mGlu5 receptors in the cerebral cortex represents a compensatory protective mechanism against SWDs that was further amplified by VU0360172. Again, experiments in which drugs are microinfused in the main stations of the cortico-thalamic-cortical network ar necessary to disclose the precise role of mGlu5 receptors in the regulation of absence seizures. The evidence that a selective mGlu5 receptor enhancer signifi- cantly reduced absence seizures in WAG/Rij rats without affecting motor behaviour is of great interest from a therapeutic standpoint. If activation of a single receptor type is warranted, receptor enhancers are advantageous over orthosteric agonists because they recruit exclusively those receptors that are endogenously activated (i.e., they act in an activity-dependent manner). This places mGlu5 receptor enhancers in the right position as novel anti-absence drugs, which might correct the abnormalities in mGlu5 receptor function without recruiting "silent" mGlu5 receptors that could generate dose-related adverse effects. It should be highlighted that more than 20% of patients with absence epilepsy and those with atypical absence seizure are refractory to conventional medication (Mikati and Holmes, 1997; Panayiotopoulos, 1999; Ollivier et al., 2009). Speculatively, mGlu5 receptor PAMs could be developed for the treatment of patients with absence epilepsy refractory to conventional medications, since subtype selective ligands of mGlu receptors are now under Phase I and II clinical studies for the treatment of neurological and psychiatric disorders (Niswender and Conn, 2010).

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# References


Alagarsamy, S., Saugstad, J., Warren, L., Mansuy, I.M., Gereau 4th, R.W., Conn, P.J., 2005. NMDA-induced potentiation of mGluR5 is mediated by activation of protein phosphatase 2B/calcineurin. Neuropharmacology. 49, 135-145.

Anderson, J.J., Rao, S.P., Rowe, B., Giracello, D.R., Holtz, G., Chapman, D.F., Tehrani, L., Bradbury, M.J., Cosford, N.D., Varney, M.A., 2002. [3H]Methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine binding to metabotropic glutamate receptor subtype 5 in rodent brain: in vitro and in vivo characterization. J Pharmacol Exp Ther. 303, 1044-1051.

Aronica, E., Gorter, J.A., IJlst-Keizers, H., Rozemuller, A.J., Yankaya, B., Leenstra, S., Troost, D., 2003. Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. Eur J Neurosci. 17, 2106-2118.

Attucci, S., Carlà, V., Mannaioni, G., Moroni, F., 2001. Activation of type 5 metabotropic glutamate receptors enhances NMDA responses in mice cortical wedges. Br. J. Pharmacol. 132, 799-806.

Avoli, M., Gloor, P., 1982. Interaction of cortex and thalamus in spike and wave discharges of feline generalized penicillin epilepsy. Exp Neurol. 76, 196-217.

Awad, H., Hubert, G.W., Smith, Y., Levey, A.I., Conn, P.J., 2000. Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. J. Neurosci. 20, 7871-7879.

Blumenfeld, H., 2005. Cellular and network mechanisms of spike-wave seizures. Epilepsia. 46, 21-33.

Bouwman, B.M., Suffczynski, P., Midzyanovskaya, I.S., Maris, E., van den Broek, P.L., van Rijn, C.M., 2007. The effects of vigabatrin on spike and wave discharges in WAG/Rij rats. Epilepsy Res. 76, 34-40.

Burgess, D.L., Biddlecome, G.H., McDonough, S.I., Diaz, M.E., Zilinski, C.A., Bean, B.P., Campbell, K.P., Noebels, J.L., 1999. beta subunit reshuffling modifies N- and P/Q-type Ca2+ channel subunit compositions in lethargic mouse brain. Mol Cell Neurosci. 13, 293-311.

Chapman, A.G., Nanan, K., Williams, M., Meldrum, B.S., 2000. Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacology. 39, 1567-1574.

Coenen, A.M., van Luijtelaar, E.L., 1987. The WAG/Rij rat model for absence epilepsy: age and sex factors. Epilepsy Res. 1, 297-301.

Coenen, A.M., van Luijtelaar, E.L., 2003. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav Genet. 33, 635-655.

Cosford, N.D., Roppe, J., Tehrani, L., Schweiger, E.J., Seiders, T.J., Chaudary, A., Rao, S., Varney, M.A., 2003. [3H]-methoxymethyl-MTEP and [3H]-methoxy-PEPy: potent and selective radioligands for the metabotropic glutamate subtype 5 (mGlu5) receptor. Bioorg Med Chem Lett. 13, 351-354.

Doherty, A.J., Palmer, M.J., Henley, J.M., Collingridge, G.L., Jane, D.E., 1997. (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but no mGlu1, receptors expressed in CHO cells and potentiates NMDA responses in the hippocampus. Neuropharmacology. 36, 265-267.

Ferraguti, F., Crepaldi, L., Nicoletti, F., 2008. Metabotropic glutamate 1 receptor: current concepts and perspectives. Pharmacol Rev. 60, 536-581.

Inoue, M., Peeters, B.W., van Luijtelaar, E.L., Vossen, J.M., Coenen, A.M., 1990. Spontaneous occurrence of spike-wave discharges in five inbred strains of rats. Physiol Behav. 48, 199-201.

Jasper, H.H., Kershman, J., 1941. Electroencephalographic classification of the epilepsies. Arch Neurol Psychiatry, Chicago. 45, 903-943.

Lasoń, W., Przewłocka, B., Van Luijtelaar, E.L., Coenen, A.M., Przewłocki, R., 1992. Endogenous opioid peptides in brain and pituitary of rats with absence epilepsy. Neuropeptides. 21, 147-152.

Liu, X.B., Muñoz, A., Jones, E.G., 1998. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol. 395, 450-465.

Lojková, D., Mares, P., 2005. Anticonvulsant action of an antagonist of metabotropic glutamate receptors mGluR5 MPEP in immature rats. Neuropharmacology. 49, 219-229.

Lourenço Neto, F., Schadrack, J., Berthele, A., Zieglgänsberger, W., Tölle, T.R., Castro-Lopes, J.M., 2000. Differential distribution of metabotropic glutamate receptor subtype mRNAs in the thalamus of the rat. Brain Res. 854, 93-105.

Mannaioni, G., Marino, M.J., Valenti, O., Traynelis, S.F., Conn, P.J., 2001. Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J. Neurosci. 21, 5925-5934.

Mathiesen, J.M., Svendsen, N., Bräuner-Osborne, H., Thomsen, C., Ramirez, M.T., 2003. Positive allosteric modulation of the human metabotropic glutamate receptor 4 (hmGluR4) by SIB-1893 and MPEP. Br J Pharmacol. 138, 1026-1030.

Meeren, H.K., Pijn, J.P., van Luijtelaar, E.L., Coenen, A.M., Lopes da Silva, F.H., 2002. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci. 22, 1480-1495.

Mikati, M.A., Holmes, G.L., 1997. Lamotrigine in absence and primary generalized epilepsies. J Child Neurol. 1, 29-37.

Molinaro, G., Traficante, A., Riozzi, B., Di Menna, L., Curto, M., Pallottino, S., Nicoletti, F., Bruno, V., Battaglia, G., 2009. Activation of mGlu2/3 metabotropic glutamate receptors negatively regulates the stimulation of inositol phospholipid hydrolysis mediated by 5-hydroxytryptamine2A serotonin receptors in the frontal cortex of living mice. Mol Pharmacol. 76, 379-387.

Ngomba, R.T., Ferraguti, F., Badura, A., Citraro, R., Santolini, I., Battaglia, G., Bruno, V., De Sarro, G., Simonyi, A., van Luijtelaar, G., Nicoletti, F., 2008. Positive allosteric modulation of metabotropic glutamate 4 (mGlu4) receptors enhances spontaneous and evoked absence seizures. Neuropharmacology. 54, 344-354.

Ngomba, R.T., Santolini, I., Salt, T.E., Ferraguti, F., Battaglia, G., Nicoletti, F., van Luijtelaar, G., 2011a. Metabotropic glutamate receptors in the thalamocortical network: strategic targets for the treatment of absence epilepsy. Epilepsia. 52, 1211-1222.

Ngomba, R.T., Santolini, I., Biagioni, F., Molinaro, G., Simonyi, A., van Rijn, C.M., D'Amore, V., Mastroiacovo, F., Olivieri, G., Gradini, R., Ferraguti, F., Battaglia, G., Bruno, V., Puliti, A., van Luijtelaar, G., Nicoletti, F., 2011b. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology. 60, 1281-1291.

Nicoletti, F., Iadarola, M.J., Wroblewski, J.T., Costa, E., 1986. Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: developmental changes and interaction with alpha 1-adrenoceptors. Proc Natl Acad Sci U S A. 83, 1931-1935.

Nicoletti, F., Bockaert, J., Collingridge, G.L., Conn, P.J., Ferraguti, F., Schoepp, D.D., Wroblewski, J.T., Pin, J.P., 2011. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology. 60, 1017-1041.

Niswender, C.M., Conn, P.J., 2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol. 50, 295-322.

Ollivier, M.L., Dubois, M.F., Krajinovic, M., Cossette, P., Carmant, L., 2009. Risk factors for valproic acid resistance in childhood absence epilepsy. Seizure. 18, 690-694.

Panayiotopoulos, C.P., 1999. Typical absence seizures and their treatment. Arch Dis Child. 81, 351-355.

Paxinos, G., Watson, C., 2005. The Rat Brain in the Stereotaxic Coordinates. Academic Press Ltd., London.

Peeters, B.W., van Rijn, C.M., Vossen, J.M., Coenen, A.M., 1989. Effects of GABA-ergic agents on spontaneous non-convulsive epilepsy, EEG and behaviour, in the WAG/Rij inbred strain of rats. Life Sci. 45, 1171-1176.

Peeters, B.W., van Rijn, C.M., Vossen, J.M., Coenen, A.M., 1990. Involvement of NMDA receptors in non-convulsive epilepsy in WAG/Rij rats. Life Sci.47, 523-529.

Pisani, A., Gubellini, P., Bonsi, P., Conquet, F., Picconi, B., Centonze, D., Bernardi, G., Calabresi, P., 2001. Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-d-aspartate responses in medium spiny striatal neurons. Neuroscience 106, 579-587.

Polack, P.O., Mahon, S., Chavez, M., Charpier, S., 2009. Inactivation of the somatosensory cortex prevents paroxysmal oscillations in cortical and related thalamic neurons in a genetic model of absence epilepsy. Cereb Cortex. 19, 2078-2091.

Rodriguez, A.L., Grier, M.D., Jones, C.K., Herman, E.J., Kane, A.S., Smith, R.L., Williams, R., Zhou, Y., Marlo, J.E., Days, E.L., Blatt, T.N., Jadhav, S., Menon, U.N., Vinson, P.N., Rook, J.M., Stauffer, S.R., Niswender, C.M., Lindsley, C.W., Weaver, C.D., Conn, P.J., 2010. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. Mol Pharmacol. 78, 1105-1123.

Romano, C., Sesma, M.A., McDonald, C.T., O'Malley, K., Van den Pol, A.N., Olney, J.W., 1995. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J Comp Neurol. 355, 455-469.

Salt, T.E., Binns, K.E., 2000. Contributions of mGlu1 and mGlu5 receptors to interactions with N-methyl-D-aspartate receptor-mediated responses and nociceptive sensory responses of rat thalamic neurons. Neuroscience. 100, 375-380.

Schridde, U., van Luijtelaar, G., 2004. The influence of strain and housing on two types of spike-wave discharges in rats. Genes Brain Behav. 3, 1-7.

Stinehelfer, S., Vruwink, M., Burette, A., 2000. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 861, 37-44.

Snead, O.C. 3rd, Banerjee, P.K., Burnham, M., Hampson, D., 2000. Modulation of absence seizures by the GABA(A) receptor: a critical role for metabotropic glutamate receptor 4 (mGluR4). J Neurosci. 20, 6218-6224.

Sun, Q.Q., Zhang, Z., Jiao, Y., Zhang, C., Szabó, G., Erdelyi, F., 2009. Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. J Neurophysiol. 101, 2679-2692.

Tu, J.C., Xiao, B., Naisbitt, S., Yuan, J.P., Petralia, R.S., Brakeman, P., Doan, A., Aakalu, V.K., Lanahan, A.A., Sheng, M., Worley, P.F., 1999. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron. 23, 583-592.

Ugolini, A., Corsi, M., Bordi, F., 1999. Potentiation of NMDA and AMPA responses by the specific mGluR5 agonist CHPG in spinal cord motoneurons. Neuropharmacology. 38, 1569-1576.

van de Bovenkamp-Janssen, M.C., van der Kloet, J.C., van Luijtelaar, G., Roubos, E.W., 2006. NMDA-NR1 and AMPA-GluR4 receptor subunit immunoreactivities in the absence epileptic WAG/Rij rat. Epilepsy Res. 69, 119-128.

van Luijtelaar, E.L., Coenen, A.M., 1986. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett. 70, 393-397.

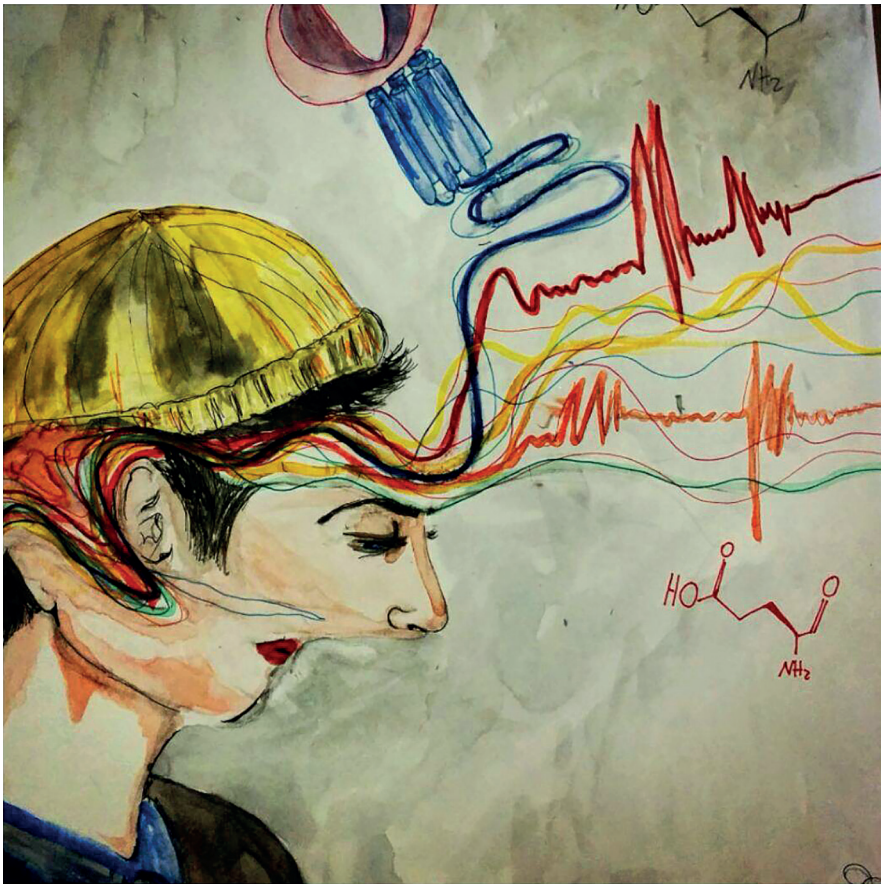
van Luijtelaar, E.L., Coenen, A.M., 1988. Circadian rhythmicity in absence epilepsy in rats. Epilepsy Res. 2, 331-336.

van Luijtelaar, G., Sitnikova, E., 2006. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 30, 983-1003.

van Rijn, C.M., Gaetani, S., Santolini, I., Badura, A., Gabova, A., Fu, J., Watanabe, M., Cuomo, V., van Luijtelaar, G., Nicoletti, F., Ngomba, R.T., 2010. WAG/Rij rats show a reduced expression of CB1 receptors in thalamic nuclei and respond to the CB1 receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. Epilepsia. 51, 1511-1521.

Wijetunge, L.S., Till, S.M., Gillingwater, T.H., Ingham, C.A., Kind, P.C., 2008. mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. J Neurosci. 28, 13028-13037.

Zhang, L., Jones, E.G., 2004. Corticothalamic inhibition in the thalamic reticular nucleus. J Neurophysiol. 91, 759-766.



# 4 Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats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# Abstract

Acute treatment with positive allosteric modulators (PAMs) of mGlu1 and mGlu5 metabotropic glutamate receptors (RO0711401 and VU030172, respectively) reduces the incidence of spike-and-wave discharges in the WAG/Rij rat model of absence epilepsy. However, from a therapeutic standpoint, it is important to establish whether tolerance develops to this antiepileptic activity after chronic administration. We therefore injected either VU0360172 (3 mg/kg, s.c.) or RO0711401 (10 mg/kg, s.c.) into WAG/Rij rats twice daily for ten days. VU0360172, maintained most of its antiepileptic activity during the ten days of treatment, whereas rats developed tolerance to RO0711401 by the end of the 3$^{rd}$ day of treatment and remained refractive for two days after treatment withdrawal. Immunoblot analysis revealed that, in response to VU0360172, expression of mGlu5 receptors increased in the thalamus of WAG/Rij rats after 1 day of treatment, and remained elevated afterwards. VU0360172 also enhanced mGlu5 receptor expression in the cortex after 8 days of treatment without affecting the expression of mGlu1α receptors. Treatment with RO0711401 enhanced the expression of *both* mGlu1α and mGlu5 receptors in the thalamus and cortex of WAG/Rij rats after 3-8 days of treatment. These data were different from those obtained in non-epileptic control rats, where repeated injections with VU0360172 and RO0711401 down-regulated the expression of mGlu1α and mGlu5 receptors. Finally, brain levels of both compounds remained unaltered after repeated administration, although, there was a decrease of cortical levels of RO0711401 from day 3 to day 8. In conclusion, mGlu5 receptors PAMs are suitable candidates for the development of novel anti-absence drugs in humans.

***Key words*:** Absence Epilepsy; Spike-Wave Discharges; WAG/Rij rats; VU0360172; RO0711401; tolerance; chronic treatment; group I mGlu receptors.

# 1. Introduction

About 20% of patients with absence epilepsy, particularly those with atypical absence seizures, are refractory to the currently used antiabsence drugs, such as ethosuximide, valproate, and clonazepam (Panayiotopoulos et al., 1999; Glauser et al., 2010). Moreover, in patients that are not refractory, antiepileptic drugs may cause class-related adverse effects, such as sedation, dizziness, amnesia and ataxia. Unfortunately, newer anti-absence drugs, such as lamotrigine and lacosemide, loose efficacy during chronic treatment (Glauser et al., 2010) or may have only marginal effects or paradoxically enhance absences seizures in animal models (van Rijn et al., 1994; van Luijtelaar et al., submitted). Therefore, more effective antiepilepsy drugs are still needed.

Metabotropic glutamate (mGlu) receptors are potential targets for novel anti-absence drugs. mGlu receptors form a family of eight, G-protein-coupled subtypes (mGlu1-8). mGlu1 and mGlu5 receptors are coupled to Gq/G11, whereas the rest are coupled to Gi/Go. These receptors are strategically distributed at synapses of the cortico-thalamo-cortical network, which is the anatomical site of origin of spike-wave discharges (SWDs) underlying absence epilepsy (Ngomba et al., 2011a; van Luijtelaar et al., 2011). For example, mGlu1 and mGlu5 receptors are localized postsynaptically on neurons of ventrobasal thalamic nuclei (VB) (Romano et al., 1995; Liu et al., 1998), whereas mGlu4 receptors are localized presynaptically on cortical glutamatergic neurons afferent to the thalamic reticular nucleus (Ngomba et al., 2008). In the cerebral cortex, mGlu1 receptors are found on GABAergic interneurons (Stinehelfer et al., 2000), whereas mGlu5 receptors are expressed by pyramidal neurons postsynaptic to thalamo-cortical projections (Wijetunge et al., 2008), as well as by interneurons. The study of mGlu receptors in models of absence epilepsy has been facilitated by the recent availability of potent and systemically active subtype-selective ligands. Noteworthily, some of these drugs are now under clinical development for neurological and psychiatric disorders showing an overall good profile of safety and tolerability (reviewed by Nicoletti et al., 2011).

Ligands that bind to the glutamate recognition site of mGlu receptors behave as orthosteric agonists or antagonists. On the other hand, ligands that bind to an allosteric site typically localized on the seven-transmembrane domain, can act as either positive or negative allosteric modulators (PAMs and NAMs, respectively). By definition, PAMs amplify receptor function only in the presence of an orthosteric agonist, and therefore recruit only mGlu receptors activated by endogenous glutamate (reviewed Niswender and Conn, 2010). Acute systemic treatment with mGlu1 and mGlu5 receptor PAMs (RO0711401 and VU030172, respectively) reduces the incidence of SWDs in the WAG/Rij rat model (Ngomba et al., 2011b; D'Amore et al. 2013), a genetic model of absence epilepsy with excellent predictive validity (Coenen and van Luijtelaar, 2003; van Luijtelaar and Sitnikova, 2006). Because RO0711401 and VU030172 do not affect motor behavior, there are interesting for the development as anti-absence drugs for humans. However, from a therapeutic

standpoint it is important to establish whether the anti-absence activity of mGlu1 and mGlu5 receptors PAMs is retained or not in response to repeated drug administrations. In fact, the development of tolerance seriously limits the therapeutic use of clonazepam and other benzodiazepines currently used in the treatment of epilepsy (Peeters et al., 1990).

# 2. Materials and Methods

## 2.1. Drugs

VU0360172 (N-clyclobutyl-6-[2-3(fluorophenyl) ethynyl] pyridine-3-carboxamine), a selective mGlu5 receptor PAM, was obtained from Vanderbilt University Medical Center (Williams et al. 2011). RO0711401 (9H-xanthene-9-carboxylic acid (4-trifluoromethyl-oxazol-2-yl) amide, a selective mGlu1 receptor PAM, was kindly provided by F.Hoffmann -La Roche (Basel, Switzerland). VU0360172 was dissolved in 10% Tween 80 and injected subcutaneously (s.c.). RO0711401 was dissolved in arachis oil (Sigma-Aldrich, Italy) and also injected s.c. All drug solutions were prepared freshly daily.

Control animals received equal volumes of 10% Tween 80 or arachis oil, s.c. RO0711401 was administered at 10 mg/kg because we previously found that this dose was effective at suppressing the number and mean duration of SWDs (Ngomba et al., 2011b). VU0360172 was administered at the centrally active dose of 3 mg/kg (Rodriguez et al., 2010), which was also effective in reducing SWD number and mean duration (D'Amore et al. 2013), taken into consideration that very high doses (30 mg/kg and more) of mGlu5 PAMs [e.g. 5PAM523 = (4-Fluorophenyl){(2R,5S)-5-[5-(5-fluoropyridin-2-yl)-1,2,4-oxadiazol-3-yl]-2-methyl-piperidin-1-yl}methanone), has been reported to show neurotoxic and seizure side effects in Female Wistar Hannover rats (Parmentier-Batteur et al 2013).

## 2.2. Animals

Thirty-six male WAG/Rij and six male ACI (Agouti Copenhagen Irish) rats were used for combined EEG–behavioral studies. The rats were born and raised at Radboud University Nijmegen, The Netherlands, and had a mean body weight of 350 g at 9 months of age. They were defined as being "symptomatic" at 6 months of age, when they develop about 16–20 SWDs per hour, or more than 200 SWDs per day (van Luijtelaar and Coenen, 1988). The animals were individually housed in Macrolon cages, kept under controlled conditions (20°C, 60% humidity) in a room with a reversed light–dark cycle (white light on from 9 p.m. to 9 a.m.), with food and drinking water always available. Animals were handled regularly a few days before starting EEG registrations.

In addition, twelve 3-month-old male Wistar rats (mean body weight, 200–240 g; Charles River, Italy) and five 3 to 4-month-old male Wistar rats (mean body weight, 380 – 420 g; Charles River, Italy) were used for Western blot analysis and EEG recording

respectively. These rats were housed in similar conditions of WAG/Rij and ACI rats in the animal facility at Neuromed Pozzilli, Italy. Male adult cervelet-4 (crv4) homozygous mutant mice lacking mGlu1 receptors (Conti et al., 2006; kindly provided by Prof. Alda Maria Puliti, University of Genoa, Italy) and male adult mGlu5-/- mice (breeded at Neuromed Institute) were used to test the specificity of the antibodies used for the detection of mGlu1α and mGlu5 receptors in Western blot experiments. The study was performed in accordance with the guidelines of the European Community for the use of experimental animals and was approved by local ethics committee for animal studies (RU-DEC). All efforts were made to reduce discomfort experienced by the animals and to keep the number of animals as low as acceptable.

## 2.3. Drug injection regimens

For EEG recordings and assessment of spontaneous motor activity, four separate groups of 8-9 WAG/Rij rats were treated twice daily (at 9 a.m. and 9 p.m.) for 10 days with RO0711401 (10 mg/kg, s.c.), VU0360172 (3 mg/kg, s.c.), or their respective vehicles (see above, s.c.). Drugs and vehicles were injected once more at 9 a.m. 2 days after withdrawal (day 13). Additional groups of WAG/Rij rats (n = 16, 4 rats per group) or non-epileptic Wistar rats (n = 12, 3 rats per group) were treated twice daily for 8 days with drugs or vehicles, and used for immunoblot analysis of mGlu1α and mGlu5 receptors in the thalamus and cerebral cortex. The same WAG/Rij rats treated with RO0711401 or VU0360172 were also used for measurements of drug levels in the thalamus, and cerebral cortex. These Wistar and WAG/Rij rats were sacrificed 1 hour after the morning injection (i.e. at 10 a.m.). Additional groups of non epileptic rats (Wistar or ACI rats) were injected with VU0360172, 3 mg/kg, s.c. or its vehicles.

## 2.4. In vivo Recordings

### 2.4.1. EEG

A cortical tripolar electrode set was implanted via stereotactic surgery under isoflurane anaesthesia supplemented with pre- and postoperative Rimadyl as analgesic and lidocaine as local anaesthetic. One active electrode was implanted in the frontal region (coordinates with the skull surface flat and from bregma zero-zero, AP +2, 0: L -3, 5) with a second one in the parietal region (A -6, 0: L -4, 0) (Paxinos and Watson, 2005). The ground electrode was placed over the cerebellum. After surgery the rats had two weeks to recover, after which, they were moved into transparent EEG recording cages supplied sawdust and cage enrichment and with water and food ad libitum. WAG/Rij and ACI rats were connected to an EEG cable with a preamplifier and a swivel, which allowed free movement. Before recording the rats were habituated to the leads for at least 12 hours. The differential recorded EEG was filtered (only frequencies between 1 and 100 Hz were allowed to pass) and were digitalized with a sample frequency of 512 Hz, and saved for an off-line analysis using Windaq system (DATAQ, Instruments, Akron, OH, USA). Wistar rats

were implanted with stainless-steel wire recording electrodes epidurally on the left and right parietal cortex under isofluorane anaesthesia supplemented with lidocaine local anaesthetic. EEG was recorded by means of Grass-Telefactor system and visually analysed to evaluate the occurrence of SWDs.

Baseline EEG recordings (Day 0) were carried out at day 0 during the first two hours of the dark period (i.e. 9 a.m. 11 a.m.). EEG and behavioural recordigs were taken during the dark-phase, five hours post injection, because this is when WAG/Rij rats have the greatest incidence of SWDs (van Luijtelaar and Coenen, 1988; Smyk et al., 2012). SWDs were labelled visually using common criteria, regular trains of sharp spikes and slow waves lasting from of 1–10 s, spike-wave frequency of 7–10 Hz, a spikes amplitude at least twice the background signal and asymmetric appearance of the SWDs (van Luijtelaar and Coenen, 1986; Ovchinnikov et al., 2010)

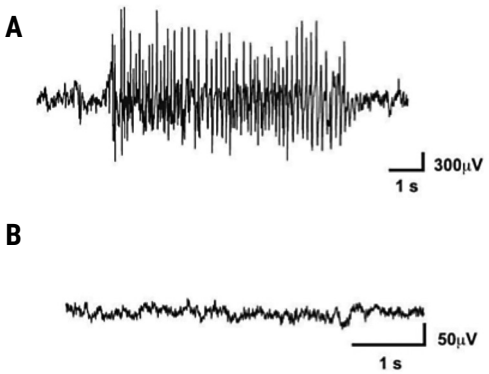


**Figure 1** Representative EEG recording from symptomaticWAG/Rij rats and age-matched Wistar rats. A typical SWDs in the EEG recording ofWAG/Rij rats is shown in (A). SWDs had a frequency of 7-10 Hz, an amplitude of at least twice the background, and a minimal duration of 1s. Note the absence of SWDs in control Wistar rats (B).

### 2.4.2. Spontaneous motor activity

Spontaneous motor activity was recorded as previously reported (van Rijn et al. 2010); an analogic passive infrared detector (PIR) (Luna PR, Rokonet Electronics LTD, Rishon Le Tzion, Israel) was fixed to a semi-open lid on top of the each rat's EEG recording cage. The analogue signal was digitalized simultaneously with the EEG signal. Movements were quantified by calculating the mean of the absolute value of the PIR signal per hour. The values of each individual rat were analysed to investigate if there were any differences in motor activity between baseline- and post injection periods to see if there were any drug effects.

## 2.5. Detection of RO0711401 and VU0360172 in cortex and thalamus of WAG/Rij rats

### 2.5.1. Sample Preparation

WAG/Rij rats treated with RO0711401 or VU0360172 were killed by decapitation on days 1, 3 or 8 as described above. The brains were rapidly removed, and the left portion of the thalamus and cerebral cortex were dissected and immediately frozen at -80°C. Tissue samples were homogenized with 1 ml of 0, 1% aqueous formic acid. The weight of each sample was recorded. 30 µl of tissue homogenate was added to 150 µl of internal standard working solution (1mM Dansilnorvaline in 100% acetonitrile). After extensive vortex (60 sec), samples were centrifuged at 14,000 rpm for 5 min. 40 µl of supernatant was then mixed with 160 µl of 0.1% aqueous formic acid and transferred to an autosampler vial for injection into the chromatographic system.

### 2.5.2 Liquid Chromatography–Tandem Mass Spectrometry Analysis

HPLC analysis was performed with an Agilent Liquid Chromatography System series 1100 (Agilent Technologies, USA), with a binary pump, an autosampler, a solvent degasser and a column oven. Chromatographic separation was performed with a 50×2.0 mm, Luna C18, 5µm, 100 Å pore size column (Phenomenex, Torrance, CA, USA), equipped with a Security guard precolumn (Phenomenex, Torrance, CA, USA), containing the same packing material. The column was maintained at room temperature. The mobile phase was a solution of 0.1% aqueous formic acid (eluent A) and 100% acetonitrile (eluent B). The injection volume was 20 µl; elution was performed at a flow rate of 300 µl/min, using 10% solvent B for 1 minute, linear gradient to 90% solvent B for 3 min, 90% solvent B for 2 min and afterwards re-equilibrating with 90% solvent A for 4 min. The total analysis run time was 10 min.

Mass spectrometry was performed with a 3200 Triple Quadrupole system, equipped with a Turbo Ion Spray source (Applied Biosystems, Foster City, CA). Data were acquired and processed with Analyst 1.4.2 software. The detector was set in the positive ion mode. The ion spray voltage was set at 5000 V and the source temperature was 300°C. The collision activation dissociation (CAD) gas was set at medium value and nitrogen was used as the collision gas. The Q1 and Q3 quadrupoles were tuned for the unit mass resolution. The transitions of the precursor ions to the product ions were monitored with a dwell time of 100 ms for each analyte. The instrument was set in the multiple reaction monitoring (MRM) modes and mass spectrometer parameters were optimized to maximize sensitivity for all transitions.

Data analysis were carried out using a calibration curve with established known concentrations (0, 6.25, 12.5, 25, 50, 100, 250, 500, and 1000 ng/ml) of RO0711401 and VU0360172 dissolved in 0.1% aqueous formic acid and processed in the same way as of tissue samples. The equation of linear regression obtained for this value range was $y = 0.000204x + 0.0033$ ($r = 0.9982$). Mass spectrometer parameters were optimized to maximize sensitivity for each transition of both drugs (internal standard) and the instrument was set in the multiple-reaction monitoring mode.

## 2.6 Western blot analysis of mGlu1α and mGlu5 receptors

This analysis was carried out in the right portions of the thalamus and cerebral cortex dissected from WAG/Rij and Wistar rats treated for 1, 3 or 8 days with RO0711401, VU0360172, or their respective vehicles. Tissue samples were homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Proteins were resuspended in SDS-bromophenol blue reducing buffer with 40 mM DTT. Western blot analyses were carried out using 8% SDS polyacrylamide gels, which were electroblotted onto immunoblot PVDF membranes (BioRad, Milano, Italy); filters were blocked overnight in TTBS buffer containing 5% non-fatty milk.

Specific rabbit polyclonal antibodies for mGlu1α or mGlu5 receptor were used (1:500 and 1:1,000, respectively, Upstate Biotechnology, Lake Placid, NY, USA). Blots were incubated for 1 h with primary polyclonal antibodies or a mouse monoclonal antibody to label b-actin (1:100,000, Sigma, St.Louis, MO, USA) and then incubated for 1 hour with secondary antibodies (peroxidase-coupled anti-rabbit or anti-mouse, Amersham, Piscataway, NJ, USA) diluted 1:7000 with TTBS. Immunostaining was revealed by enhanced ECL (Amersham, Piscataway,NJ,USA).

## 2.7. Statistical analysis

It was first tested whether the two vehicles differed from each other or were associated with hourly or daily EEG or behavioural parameters. Since this was not the case, it was decided to pool the data of the two control groups. The effects of chronic administration of VU0360172 or RO0711401 on incidence and mean duration of SWDs as a percentage of the base-line data as well as the behavioral activity of animals were tested in three separate repeated-measures mixed-design ANOVA with incidence and mean duration of SWDs or amplitude of the PIR as dependent variables. For all three analyses, the time of EEG recording (5 hours) and day of experiment (day 1, day 10, day 13) were used as the within-subjects factors, the administered compound (vehicle(s), VU0360172 and RO0711401) was used as the between-subjects factors. Bonferroni's test was used to isolate the differences between drugs, days and hours. Two-way mixed-design ANOVA was used for the analyses of the day effect across all injection days (1-10 and 13) in order to establish more clearly the development of tolerance and the effects of interruption of treatment. The incidence in the first hour post-drug was the dependent variable.

Statistical analysis of HPLC and Western blot data for thalamus and cortex separately were evaluated using a one-way ANOVA with drug treatment between days (day 1, day 3, day 8) as within-subjects factor, followed by the post-hoc analysis (Bonferroni's multiple comparison test). Data are expressed as means ± SEM. Where values are means ± S.E.M. of 3 determinations per group with *$p<0.05$ vs. Control and # $p<0.05$ vs. day 1 or § $p<0.05$ vs. day 3 treatment.

# 3. Results

## 3.1. Acute and chronic administration of mGlu1 and mGlu5 receptor PAMs in WAG/Rij rats: effects on SWDs

Symptomatic 6-7 months old WAG/Rij rats were treated twice daily for 10 days with RO0711401 (10 mg/kg, s.c.), VU0360172 (3 mg/kg, s.c.) or their respective vehicles. SWDs were analysed every day during the treatment and then after 3 days of withdrawal in response to drug rechallenge. Sample records of EEG activities from a representative WAG/Rij rat and a non epileptic Wistar rat are shown in Fig. 1A,B.

The analysis of the incidence of SWDs during the first 5 hours post injection (data on incidence and duration for day 1 and 10 are shown in Fig. 2) showed a drug effect ($F=14.12$, df 1,24, $p<.001$, $\eta^2=.54$; VU0360172 and RO0711401 groups had less SWDs than vehicle), an "hour" effect ($F=22.40$, df 4,96, $p<.001$, $\eta^2=.48$; hour 1 < hours 2-5) and interactions between hour x drug ($F=7.13$, df 8,96, $p<.001$; $\eta^2=.38$) and between day x drug ($F=10,20$, df 4,48, $p<.001$, $\eta^2=.46$). Post hoc analysis on day 1 showed that RO0711401 and VU0360172 reduced the incidence of SWDs in the 1st post injection hour (Fig. 2A), with VU0360172 being more effective than RO0711401. WAG/Rij rats treated with VU0360172 (but not with RO0711401) also showed a trend to a reduction in the incidence of SWDs at the 2nd hour post injection ($p>0.05$). Post-hoc analyses on the incidence of SWDs on day 10 showed that VU0360172 retained its anti-absence activity at the 1st hour post injection whereas RO0711401 became inactive. Fig. 2B shows the incidence of SWDs on day 10 of treatment. Post hoc analyses on day 13 showed exactly the same group differences as on day 10, with VU0360172, but not RO0711401, being effective as an anti-absence drug in the 1st hour post-injection. However, RO0711401 significantly reduced the incidence of SWDs in the second hour post injection on day 13 as compared to VU0360172 and vehicle ($p<0.05$; not shown), suggesting that RO0711401 had regained some of its effects after 3 days of withdrawal.

Analysis of mean duration of SWDs at days 1, 10, and 13 showed a day effect ($F=9.39$, $df=2,46$, $p<.000$, $\eta^2=.290$) and a significant second-order interaction between day x hour x drug ($F=3.54$, $df=16,184$, $p<0.000$, $\eta^2=.235$). Post hoc tests on day 1 showed that mean duration of SWDs was reduced by VU0360172 at the 2nd hour post injection (see Fig. 2C) compared to all other groups. VU0360172 had lost its effect on the mean duration of SWD at day 10 and 13. Mean duration was not affected by treatment with RO0711401 (Fig. 2C,D). To exclude that SWDs data could be biased by a potential behavioural effect of the two drugs, motor behaviour was measured with PIR at day 1, 10 and 13 during the first 5 hours post injection. Statistical analysis showed neither drug effect nor interactions effects between drug and any other independent variable (Fig. 2E,F).

The incidence of SWDs and their mean duration were exclusively analysed at 1 hour post injection on all treatment days in order to inspect the changes in drug efficacy over time. Two-way ANOVA analysis showed a drug effect ($F=591,90$, df 2,15, $p<0.001$, $\eta^2=.99$;

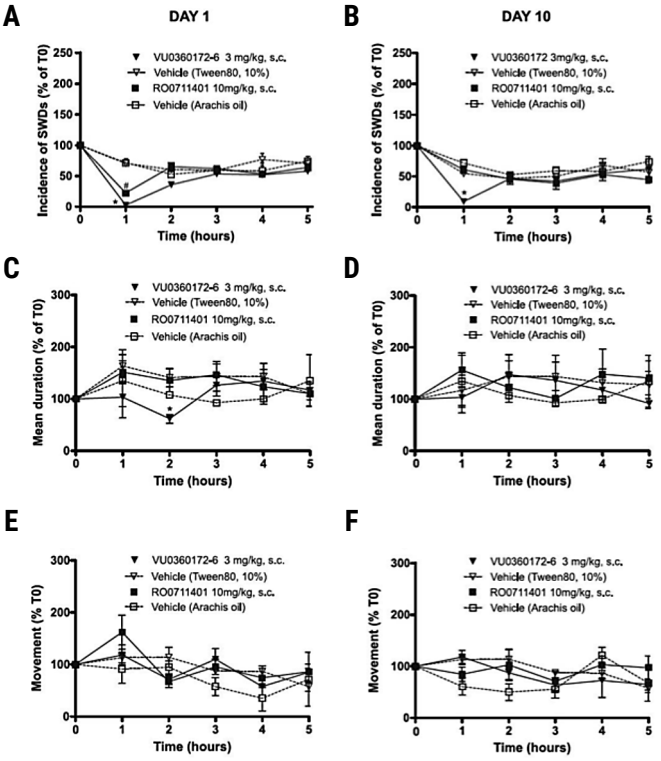


**Figure 2** Differential effects of RO0711401 and VU0360172 on the incidence of SWDs on days 1 and 10 of treatment in WAG/Rij rats. Rats were treated s.c. twice daily with RO0711401 (10 mg/kg), VU0360172 (3 mg/kg) or their respective vehicles. SWD incidence measured at baseline (time 0) and in the first 5 h post injection on day 1 and 10 of treatment are shown in (A) and (B), respectively. Mean duration of SWDs is shown in (C) and (D); spontaneous motor activity is shown in (E) and (F). Values are means + S.E.M. $P < 0.05$ vs. The respective vehicles (*) or vs. VU0360172 (#). Statistical analysis is detailed in the Results session.

VU0360172<RO0711401<vehicle), a day effect ($F=7.63$, df 10,150, $p<0.001$, $\eta^2=.34$) and an interaction between day x drug ($F=9.80$, df 20,150, $p<0.001$, $\eta^2=.57$). Post hoc analysis showed that VU0360172 retained its anti-absence action across all 10 days of treatment, and kept also its antiabsence action at the re challenge day after 2 withdrawal days. In contrast, RO0711401 showed its antiabsence effect only during the first 2 days of treatment, after that, the antiabsence action was completely and immediately lost and at day 3 the incidence of SWDs was no longer different from solvent. The anti-absence activity of RO0711401 was not restored after 2 days of drug withdrawal ($p>0.05$; Fig. 3).

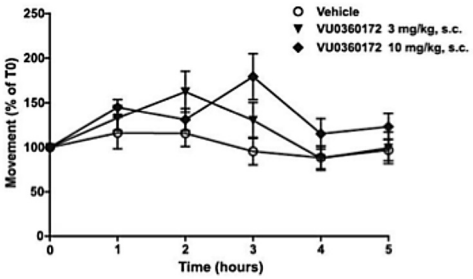


**Figure 3** Development of tolerance to the anti-absence activity of RO0711401 after the first 3 days of treatment. WAG/Rij rats were treated s.c. twice daily for 10 days with RO0711401 (10 mg/kg), VU0360172 (3 mg/kg), or the respective vehicles. A rechallenge with each of the two drugs was performed after 3 days of withdrawal. The incidence of SWDs at 1 h after the morning injection is shown. Values are means + S.E.M. $P < 0.05$ vs. the respective vehicles (*) or vs. VU0360172 (#).



There was no effect of the two drugs on the mean duration of SWDs at 1 hour post injection at any day of the treatment (not shown).

## 3.2 Measurements of thalamic and cortical drug levels during repeated administrations of RO0711401 or VU0360172

To examine whether the development of tolerance to RO0711401 was associated with time-dependent changes in central bioavailability of the drug, we measured RO0711401 levels in the thalamus and cortex in three separate groups of WAG/Rij rats treated with the drug as mentioned above and killed at day 1, 3 or 8 at one hour after the morning injection. One-way ANOVA did not reveal any significant effect of day of treatment in the thalamus (Fig.4B, $p > 0.05$) while a significant day effect was observed in the cortex (Fig.4A, $F=1,501$, $df= 1,4$, $p<0.05$, $\eta^2=.273$), with levels of RO0711401 being reduced on day 8 of treatment. However, RO0711401 levels were unchanged on day 3 of treatment, when tolerance to the anti-absence effect begun to develop (Fig.4A). We also measured VU0360172 levels and found no significant day effect, neither in cortex nor in thalamus (Fig. 4C,D, $p >0.05$).

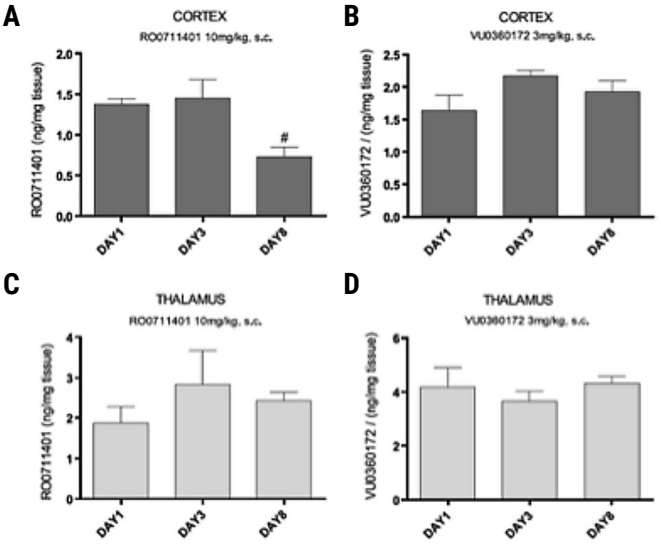


**Figure 4** Drug levels in the thalamus and cortex during 8-day treatment with RO0711401 or VU0360172 in WAG/Rij rats. Rats were treated s.c. twice daily for 8 days with RO0711401 (10 mg/kg) or VU0360172 (3 mg/kg). Values are means + S.E.M. $^{\#}P < 0.05$ vs. the respective values on day 1 or 3 of treatment.

## 3.3 Measurements of mGlu1α and mGlu5 expression in the thalamus and somatosensory cortex after treatment with RO0711401 or VU0360172

Next it was examined whether repeated administrations of RO0711401 or VU0360172 induced any adaptive changes in the expression of mGlu1α and mGlu5 receptors in WAG/Rij rats that could correlate with the temporal profile of drug response on SWD incidence.

Immunoblot analysis revealed bands at 140kDa and 130 kDa, which, that correspond to the deduced molecular sizes of the receptor's monomers (arrowheads in the representative immunoblots). A faint higher molecular weight band corresponding to dimers of mGlu1α and mGlu5 receptors was also visible in some of the immunoblots (not shown) but was not included in our densitometric analysis. The specificity of the antibodies was confirmed using the cerebellum of *crv4* mutant mice hypomorphic for mGlu1 receptors (Conti et al., 2006) or the cerebral cortex of mGlu5 receptor knockout mice as negative controls. The lower band present in the repre-sentative mGlu1a and mGlu5 immunoblots was non-specific because it was still visible in samples from crv4 and mGlu5-/- mice, respectively (Fig. 5). Treatment of WAG/Rij rats with vehicle twice daily for 8 days did not change the expression of mGlu1α and or mGlu5 receptors in the

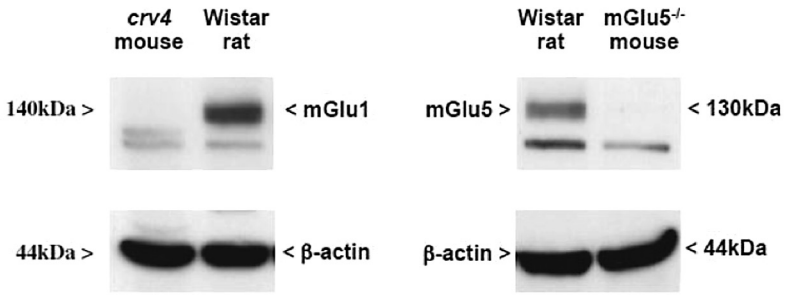


**Figure 5** Specificity of the antibodies used for immunoblot analysis of mGlu1a and mGlu5 receptors. Western blot analysis was performed in protein extracts from the cerebellum of crv4 mutant mice (left) and the cerebral cortex of mGlu5_/_ mice (right) to verify the identity of the bands corresponding to mGlu1α and mGlu5 receptors, respectively. mGlu1a and mGlu5 receptor labelling are also shown in the cortex and thalamus of Wistar rats, respectively, for comparison. The upper band at 140 and 130 kDa corresponds to mGlu1a and mGlu5 receptor monomers, respectively. The lower band was non-specific because it was still present in crv4 and mGlu5_/_ mice.



thalamus or cortex (only values at day 8 are shown and indicated as "controls"). Treatment with RO0711401 caused an increase in *both* mGlu1α and mGlu5 receptor protein levels on day 8. The drug also enhanced mGlu1α receptor levels on day 3 in the cortex [Fig. 6A-D; mGlu1α ( $F = 10.36$, $P = 0.0039$ and $F = 15.50$, $P = 0.011$; for the thalamus and cortex respectively) and mGlu5] ($F = 7.33$, $P = 0.011$ and $F = 6.15$, $P = 0.018$; for the thalamus and cortex respectively). In contrast, treatment with VU0360172 in WAG/Rij rats caused an early and persistent increase in mGlu5 receptor expression in the thalamus (Fig. 7A: $F = 7.56$, $P = 0.0042$) a late increase of mGlu5 receptor expression in the cortex (Fig. 7B: $F = 9.17$, $P = 0.0057$) and no changes in mGlu1α receptor levels in any of the two brain regions (Fig. 7C,D).

To examine whether these adaptive changes in receptor expression represented a general response to mGlu1 and mGlu5 receptor PAMs or were peculiar to epileptic WAG/Rij rats, the analysis was extended to non-epileptic age-matched Wistar rats treated with vehicles, RO0711401 or VU0360172 for 1, 3 and 8 days. It was confirmed in these rats that treatment with RO0711401 caused changes in both mGlu1α and mGlu5 receptors whereas treatment with VU0360172 caused selective changes in mGlu5 receptors. However, the direction of these changes was opposite to that observed in WAG/Rij rats. Wistar rats treated with RO0711401 showed an early and persistent down-regulation of mGlu1α and mGlu5 receptors in the thalamus and cortex [Fig. 8A-D; mGlu1α ($F = 18.51$, $P = 0.0020$ and $F = 46.19$, $P = 0.0001$; for the thalamus and cortex respectively) and mGlu5 ($F = 10.76$,

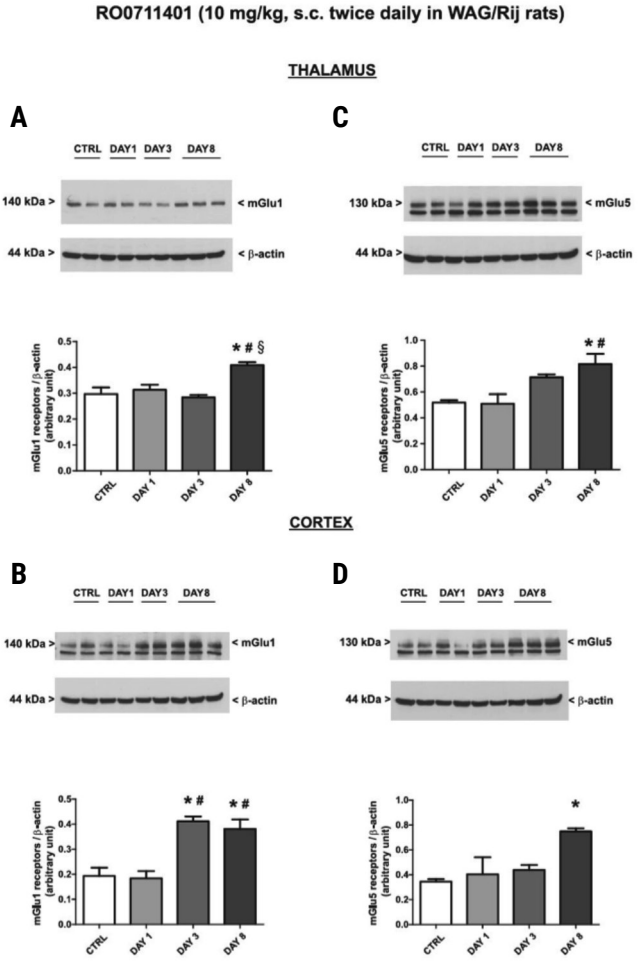


**Figure 6** Adaptive changes in the expression of mGlu1a and mGlu5 receptors caused by 8-day treatment with RO0711401 inWAG/Rij rats. Rats were treated s.c. twice daily for 8 days with RO0711401 (10 mg/kg) or the respective vehicle. Values are means + S.E.M. $P < 0.05$ (One-way ANOVA + Bonferroni's test) vs. the respective control (*), day 1 (#) or day 3 (x) values.

$P = 0.0035$ and $F = 21.00$, $P = 0.0026$; for the thalamus and cortex respectively)]. In contrast, Wistar rats treated with VU0360172 showed a late reduction in mGlu5 receptor expression in the thalamus ($F = 10.75$, $P = 0.0035$) and an early and persistent reduction of mGlu5 receptor expression in the cortex ($F = 14.82$, $P = 0.0012$) with no changes in the expression of

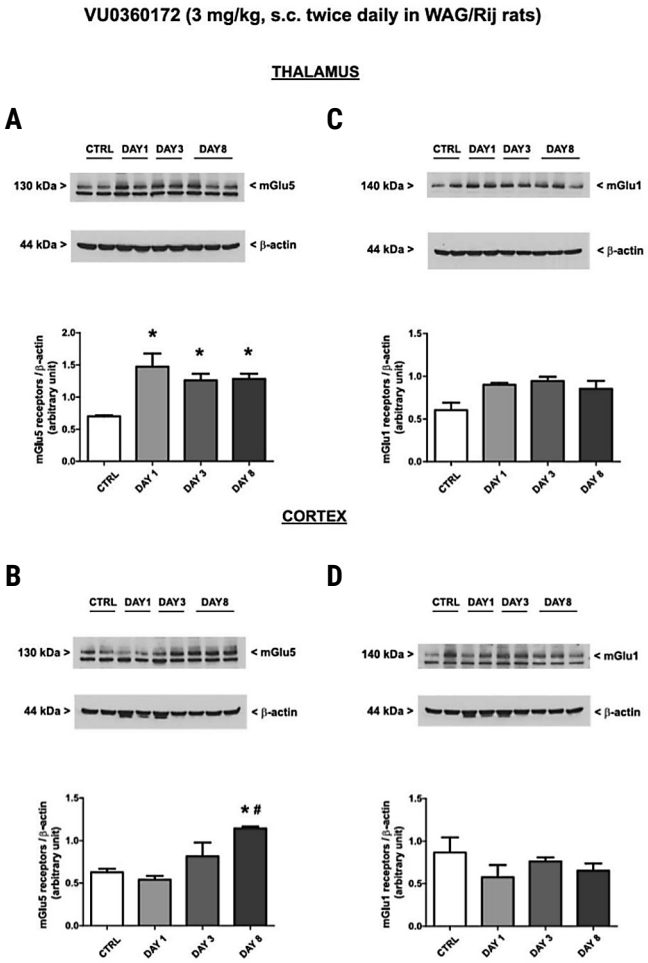


**Figure 7** Adaptive changes in the expression of mGlu5 receptors caused by 8-day treatment with VU0360172 in WAG/Rij rats. Rats were treated s.c. twice daily for 8 days with VU0360172 (3 mg/kg) or the respective vehicle. Values are means + S.E.M. $P < 0.05$ (One-way ANOVA + Bonferroni's test) vs. the respective control (*) or day 1 (#) values.

mGlu1α receptors (Fig. 9A-D). Thus, interestingly, epileptic WAG/Rij rats and non-epileptic Wistar rats showed opposite manifestations of receptor adaptation in response to prolonged mGlu1 and mGlu5 receptor enhancement. A synopsis on the effect of chronic treatment with RO0711401 or VU0360172 on the thalamic/cortical expression of mGlu1α and mGlu5 receptors is shown in Fig. 10.

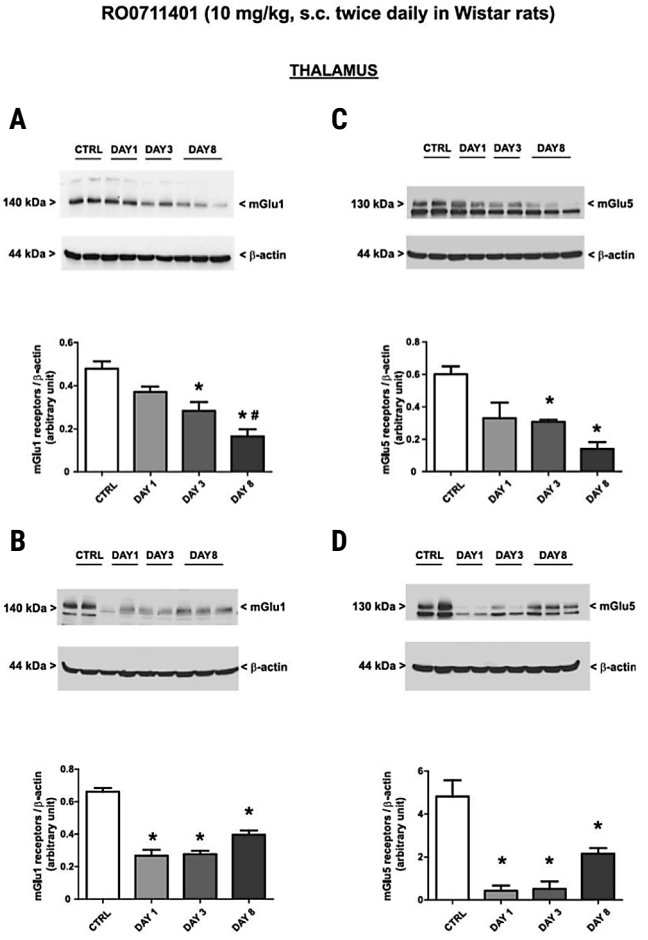


**Figure 8** Adaptive changes in the expression of mGlu1a and mGlu5 receptors caused by 8-day treatment with RO0711401 in non-epileptic Wistar rats. Rats were treated s.c. twice daily for 8 days with RO0711401 (10 mg/kg) or the respective vehicle. Values are means + S.E.M. $P < 0.05$ (One-way ANOVA + Bonferroni's test) vs. the respective control (*) or day 1 (#) values.

Finally, we examined whether chronic treatment with VU0360172 in Wistar rats could induce the appearance of SWDs as a result of the down-regulation of mGlu5 receptors in the thalamus (see D'Amore et al., 2013). No SWDs were recorded in Wistar rats treated for 8 days with VU0360172 (3 mg/kg, s.c. twice daily; EEG recorded at day 1, 3 and 8 of

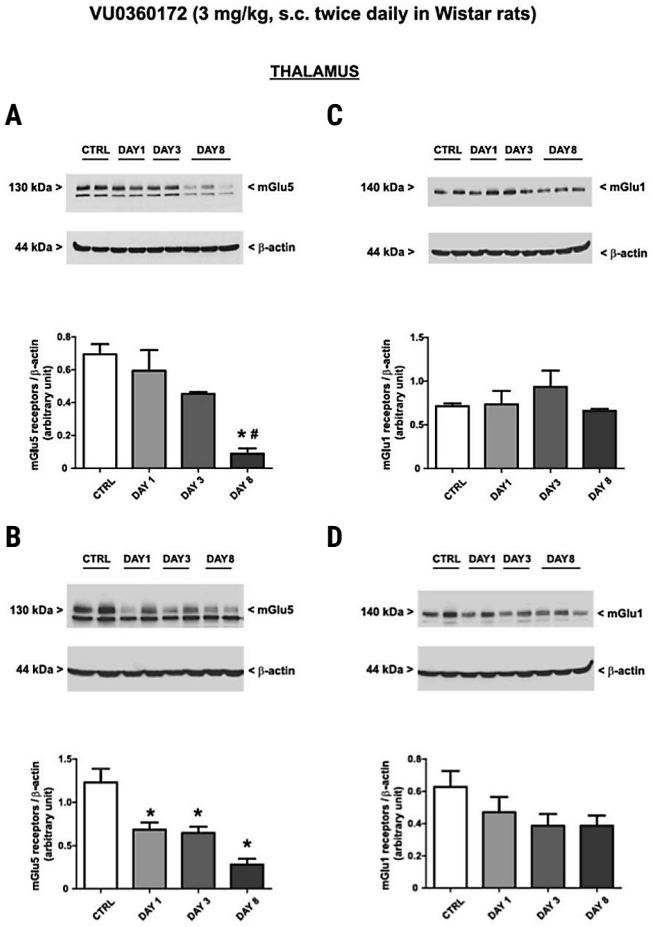


**Figure 9** Adaptive changes in the expression of mGlu5 receptors caused by 8-day treatment with VU0360172 in non-epileptic Wistar rats. Rats were treated s.c. twice daily for 8 days with VU0360172 (3 mg/kg) or the respective vehicle. Values are means + S.E.M. $P < 0.05$ (One-way ANOVA + Bonferroni's test) vs. the respective control (*) or day 1 (#) values.

treatment). Similarly, no SWDs were induced by chronic VU0360172 (3 mg/kg, s.c., twice daily for 10 days; EEG recorded at days 1, 5, and 10) in non-epileptic ACI rats, used as additional controls (representative EEG recordings shown in Fig. 11).

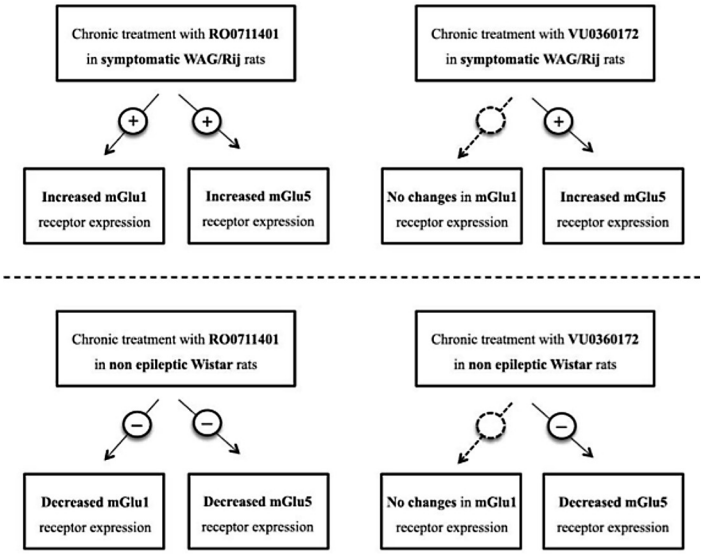


**Figure 10** Schematic diagram illustrating mGlu1/mGlu5 receptor responses in symptomatic WAG/Rij and non-epileptic control Wistar rats following chronic treatment with either mGlu1 or mGlu5 receptor PAMs. Detailed results of western blot analysis of mGlu1 and mGlu5 receptors are provided in Figs. 6-9.

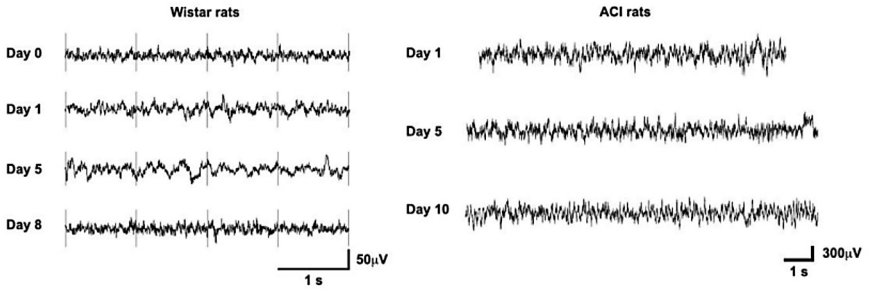


**Figure 11** EEG records showing no SWDs in Wistar or ACI rats treated chronically with VU0360172. Wistar or ACI rats (n = 3- 6 per group) were treated with VU0360172 (3 mg/kg s.c.) or vehicle twice daily for 8- 10 days, and recorded at baseline (day 0) and at the indicated days of treatment. These treatments did not cause the appearance of SWDs in the two strains of rats. Representative EEG recordings of rats treated with VU0360172 are shown.

# 4. Discussion

The main objective of the study was to establish whether tolerance develops to the SWD-suppressing activity of mGlu1 and mGlu5 receptor PAMs and to unravel the molecular nature of tolerance (if any). This is a necessary step for the preclinical and clinical development of mGlu receptor PAMs for the treatment of absence epilepsy in humans. As expected (Ngomba et al., 2011b, D'Amore et al., 2013), both mGlu1 and mGlu5 receptor PAMs were able to suppress SWDs in WAG/Rij rats in the early phases of the treatment. However, a large difference between the two drugs was apparent when the response was monitored on a daily basis during chronic administration. The anti-absence effect of the mGlu5 receptor PAM, VU0360172, on the incidence of SWDs largely persisted over time, with only small signs of tolerance. More precisely, treatment with VU0360172 caused a significant reduction in the incidence of SWDs at the 1$^{st}$ hour and a reduction trend at the 2$^{nd}$ hour on day 1. The effect at the 1$^{st}$ hour was fully maintained on day 10. On the other hand, the mean duration of SWDs was reduced at the 2$^{nd}$ hour post injection on day 1 but not on day 10. In contrast, the mGlu1 receptor PAM, RO0711401, lost its anti-absence effects on the incidence of SWDs on day 10. In order to disclose when precisely tolerance to RO0711401 began to develop, we analysed the incidence of SWDs in the first hour of injection of all ten consecutive days of treatment. The outcomes showed that the anti-absence action of RO0711401 remained unchanged from day 1 to day 2, diminished on day 3, and completely disappeared on day 4, remaining absent throughout the rest of the experiment. Therefore, tolerance to RO0711401 began to develop after relatively short time, i.e., after only four injections and was complete after two additional injections. The small and incomplete tolerance to the mGlu5 receptor PAM, VU0360172, is somehow consistent with data obtained with CDPPB another mGlu5 receptor PAM, in a mouse model of audiogenic seizures (Pacey et al., 2011). Therefore, it seems that the two drugs differ with respect to the development of tolerance, with VU0360172 retaining most of the "therapeutic" effect when repeatedly administered to WAG/Rij rats, and RO0711401 inducing fast and complete tolerance to the anti-absence effect. We have collected only few pharmacokinetic data on the two PAMs in this study. VU0360172 shows high plasma protein-binding and little or no first-pass metabolism (Rodriguez et al., 2010). R00711401 has a short elimination half-life (< 2 hours), but no data on liver metabolism are available (Vieira et al., 2009). Whether or not the two drugs may inhibit or accelerate their own metabolism with time is unknown. We found that thalamic levels of the two drugs remained stable during eight consecutive days of treatment in WAG/Rij rats, while a decrease over the days (day 3 vs. day 8) found in the cortex might indicate a contribution of pharmacokinetic tolerance to the loss of effects of RO0711401 on day 8 (but not on day 3). We next measured the expression of mGlu1$\alpha$ and mGlu5 receptors in the thalamus and cortex of WAG/Rij rats on different days of drug treatment, as compared to treatments with the respective vehicles. This analysis was also carried out on non epileptic Wistar rats

treated with the two drugs. We were surprised to find that adaptive changes in receptor expression induced by the two PAMs were highly divergent between WAG/Rij and Wistar rats. In Wistar rats, both drugs substantially reduced the expression of the respective mGlu receptor subtype particularly in the cerebral cortex. Interestingly, repeated administrations of RO0711401 also reduced the expression of the mGlu5 receptors whereas VU0360172 has no effect on the expression of the mGlu1$\alpha$ receptors. This particular type of receptor regulation was completely disrupted in WAG/Rij rats, where the two PAMs rather *enhanced* the expression of the respective subtype, although with different temporal and regional profile. However, also in this case, RO0711401 changed the expression of both the mGlu1$\alpha$ and the mGlu5 receptors, whereas VU0360172 selectively changed the expression of the mGlu5 receptors only.

In an attempt to explain the different receptor selectivity in the adaptive changes induced by the two PAMs, we wish to highlight that mGlu receptor subtypes can form both homodimers (e.g., either mGlu1-mGlu1 or mGlu5-mGlu5 dimers) and intra-group heterodimers (e.g., mGlu1-mGlu5 heterodimers), at least in heterologous expression systems (Doumazane et al., 2010). In addition, only one PAM molecule per dimer is sufficient to fully amplify receptor activity (Kniazeff et al., 2004). We speculate that in the thalamus and cortex of both WAG/Rij and Wistar rats there is a prevalence of mGlu1-mGlu1 homodimers and mGlu1-mGlu5 heterodimers over mGlu5-mGlu5 homodimers. This explains why the mGlu1 receptor PAM, RO0711401, induced adaptive changes in both mGlu1 and mGlu5 receptors, whereas the mGlu5 receptor PAM, VU0360172, selectively induced changes in mGlu5 receptors. The reason why receptor regulation caused by chronic PAM treatment is qualitatively different in WAG/Rij rats is unknown. Under normal conditions, prolonged agonist exposure causes mGlu receptor desensitization (i.e., uncoupling from G proteins), followed by receptor internalization that may lead to reducing receptor expression (i.e., receptor down-regulation) if *de novo* synthesis of receptor protein does not compensate for receptor degradation (for reviews, see Reiter amd Lefkowitz, 2006; Whalen et al., 2011). It is possible that mechanisms that lie at the core of receptor desensitization and down-regulation are distorted in WAG/Rij rats because of the hypersynchronous activity of the cortico-thalamic-cortical network. This encourages the study of molecules involved in desensitization and internalization of mGlu1 and mGlu5 receptors, such as G-protein coupled receptor kinases and $\beta$-arrestin (reviewed by Iacovelli et al., 2013) in WAG/Rij rats and other animal models of absence epilepsy.

In conclusion, our data support the development of mGlu5 receptor PAMs as potential symptomatic drugs for the chronic treatment of absence epilepsy. These drugs may not cause pharmacodynamic tolerance perhaps because they fail to induce adaptive changes in the cognate mGlu1 receptor. We wish to highlight that VU0360172 (and also the mGlu1 PAM, RO0711401) did not affect motor behaviour and did not cause gross signs of toxicity in either WAG/Rij or Wistar rats. mGlu5 receptor PAMs are already under development for the treatment of schizophrenia with the only concern of neurotoxicity

and convulsive seizures induced by very high doses of these compounds (Parmentier-Batteur et al., 2013). It remains to be established whether mGlu5 receptor PAMs can be safely associated with other drugs used in the treatment of absence epilepsy in patients that are resistant to conventional antiepileptic medication.

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# 5. References

Coenen, A. M., van Luijtelaar E. L. 2003. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav. Genet. 33, 635-655.

Conti, V., Aghaie, A., Cilli, M., Martin, N., Caridi, G., Musante, L., Candiano, G., Castagna, M., Fairen, A., Ravazzolo, R., Guenet, J. L., Puliti, A. 2006. crv4, a mouse model for human ataxia associated with kyphoscoliosis caused by an mRNA splicing mutation of the metabotropic glutamate receptor 1 (Grm1). Int. J. Mol. Med. 18, 593-600.

D'Amore, V., Santolini, I., van Rijn, C. M., Biagioni, F., Molinaro, G., Prete, A., Conn, P. J., Lindsley, C. W., Zhou, Y., Vinson, P. N., Rodriguez, A. L., Jones, C. K., Stauffer, S. R., Nicoletti, F., van Luijtelaar, G., Ngomba, R. T. 2013. Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacology 66, 330-338.

Doumazane, E., Scholler, P., Zwier, J. M., Trinquet, E., Rondard, P., Pin, J. P. 2011. A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. FASEB J. 25, 66-77.

Glauser, T. A., Cnaan, A., Shinnar, S., Hirtz, D. G., Dlugos, D., Masur, D., Clark, P. O., Capparelli, E. V., Adamson, P. C. 2010. Childhood Absence Epilepsy Study Group. Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. N. Engl. J. Med. 362, 790-799.

Kniazeff, J., Bessis, A. S., Maurel, D., Ansanay, H., Prézeau, L., Pin, J. P. 2004. Closed state of both binding domains of homodimeric mGlu receptors is required for full activity. Nat. Struct. Mol. Biol. 11, 706-713.

Iacovelli, L., Nicoletti, F., De Blasi, A. 2013. Molecular mechanisms that desensitize metabotropic glutamate receptor signaling: an overview. Neuropharmacology 66, 24-30.

Liu, X.B., Muñoz, A., Jones, E. G. 1998. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J. Comp. Neurol. 395, 450-65.

Ngomba, R. T, Ferraguti, F., Badura, A., Citraro, R., Santolini, I., Battaglia, G., Bruno, V., De Sarro, G., Simonyi, A., van Luijtelaar, G., Nicoletti, F. 2008. Positive allosteric modulation of metabotropic glutamate 4 (mGlu4) receptors enhances spontaneous and evoked absence seizures. Neuropharmacology 54, 344-354.

Ngomba, R.T., Santolini, I., Salt, T. E., Ferraguti, F., Battaglia, G., Nicoletti, F., van Luijtelaar, G. 2011a. Metabotropic glutamate receptors in the thalamocortical network: strategic targets for the treatment of absence epilepsy. Epilepsia. 52, 1211-1222.

Ngomba, R.T., Santolini, I., Biagioni, F., Molinaro, G., Simonyi, A., van Rijn, C. M., D'Amore, V., Mastroiacovo, F., Olivieri, G., Gradini, R., Ferraguti, F., Battaglia, G., Bruno, V., Puliti, A., van Luijtelaar, G., Nicoletti, F. 2011b. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology 60, 1281-1291.

Nicoletti, F., Bockaert, J., Collingridge, G. L., Conn, P. J., Ferraguti, F., Schoepp, D. D., Wroblewski, J. T., Pin, J. P. 2011. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60, 1017-1041.

Niswender, C. M., Conn, P. J. 2010. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu. Rev. Pharmacol. Toxicol. 50, 295-322.

Ovchinnikov, A., Lüttjohann, A., Hramov, A., van Luijtelaar, G. 2010. An algorithm for real-time detection of spike-wave discharges in rodents. J. Neurosci. Methods 194, 172-178.

Pacey, L. K., Tharmalingam, S., Hampson, D. R. 2011. Subchronic administration and combination metabotropic glutamate and GABAB receptor drug therapy in fragile X syndrome. J. Pharmacol. Exp. Ther. 338, 897-905.

Panayiotopoulos, C. P. 1999. Typical absence seizures and their treatment. Arch. Dis. Child. 81, 351-355.

Parmentier-Batteur, S., Hutson, P. H., Menzel, K., Uslaner, J. M., Mattson, B. A., O'Brien, J. A., Magliaro, B. C., Forest, T., Stump, C. A., Tynebor, R. M., Anthony, N. J., Tucker, T. J., Zhang, X. F., Gomez, R., Huszar, S. L., Lambeng, N., Fauré, H., Le Poul, E., Poli, S., Rosahl, T. W., Rocher, J. P., Hargreaves, R., Williams, T. M. 2013. Mechanism based neurotoxicity of mGlu5 positive allosteric modulators - Development challenges for a promising novel antipsychotic target. Neuropharmacology 1-13 , doi: 10.1016/j.neuropharm.2012.12.003

Paxinos, G., Watson, C., 2005. The Rat Brain in the Stereotaxic Coordinates. Academic Press Ltd., London.

Peeters, B. W., van Rijn, C. M., Nutt, D. J., Titulaer, M. N., Vossen, J. M., Coenen, A. M. 1990. Diazepam and Ro 15-1788 increase absence epilepsy in WAG/Rij rats chronically exposed to diazepam. Eur. J. Pharmacol. 178, 111-114.

Reiter, E., Lefkowitz, R. J. 2006. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. Trends Endocrinol. Metab. 17, 159-165.

Rodriguez, A. L., Grier, M. D., Jones, C. K., Herman, E. J., Kane, A. S., Smith, R. L., Williams, R., Zhou, Y., Marlo, J. E., Days, E. L., Blatt, T. N., Jadhav, S., Menon, U. N., Vinson, P. N., Rook, J. M., Stauffer, S.R., Niswender, C.M., Lindsley, C.W., Weaver, C. D., Conn, P. J. 2010. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. Mol. Pharmacol. 78, 1105-1123.

Romano, C., Sesma, M. A., McDonald, C. T., O'Malley, K., Van den Pol, A. N., Olney, J. W. 1995. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J. Comp. Neurol. 355, 455-469.

Smyk, M. K., Coenen, A., Lewandowski, M. H., van Luijtelaar, G. 2012. Internal desynchronization facilitates seizures. Epilepsia 53, 1511-1518

Stinehelfer, S., Vruwink, M., Burette, A. 2000. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 861, 37-44.

van Luijtelaar, E.L., Coenen, A. M. 1986. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci. Lett. 70, 393-397.

van Luijtelaar, E. L., Coenen, A. M. 1988. Circadian rhythmicity in absence epilepsy in rats. Epilepsy Res. 2, 331-336.

van Luijtelaar, G., Sitnikova, E. 2006. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci. Biobehav. Rev. 30, 983-1003.

van Luijtelaar, G., Sitnikova, E., Littjohann, A. 2011. On the origin and suddenness of absences in genetic absence models. Clin. EEG Neurosci. 42, 83-97.

van Luijtelaar, G., Bikbaev, A., LeClerck, K., Mantagne, A., Kaminski, R. 2013. The effects of lacosamide on absence seizures in the WAG/Rij and GAERS models. Epilepsia, submitted.

van Rijn, C. M., Weyn Banningh, E. W., Coenen, A. M. 1994. Effects of lamotrigine on absence seizures in rats. Pol. J. Pharmacol.46, 467-470.

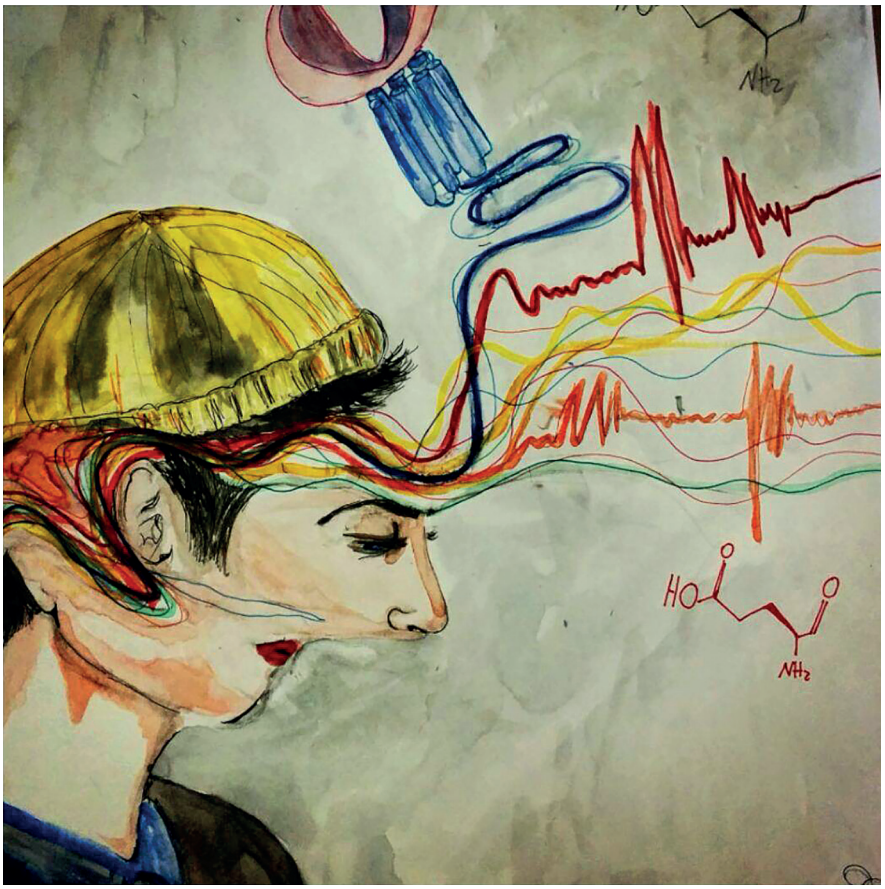
van Rijn, C. M., Gaetani, S., Santolini, I., Badura, A., Gabova, A., Fu, J., Watanabe, M., Cuomo, V., van Luijtelaar, G., Nicoletti, F., Ngomba, R. T. 2010. WAG/Rij rats show a reduced expression of CB1 receptors in thalamic nuclei and respond to the CB`1 receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. Epilepsia 51, 1511-1521.

Vieira, E., Huwyler, J., Jolidon, S., Knoflach, F., Mutel, V., Wichmann, J. 2009. Fluorinated 9H-xanthene-9-carboxylic acid oxazol-2-yl-amides as potent, orally available mGlu1 receptor enhancers. Bioorg. Med. Chem. Lett. 19, 1666-1669.

Whalen, E. J., Rajagopal, S., Lefkowitz, R. J. 2011. Therapeutic potential of β-arrestin- and G protein-biased agonists. Trends Mol. Med. 17, 126-139.

Wijetunge, L. S., Till, S. M., Gillingwater, T. H., Ingham, C. A., Kind, P. C. 2008. mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. J. Neurosci. 28, 13028-13037.

Williams, R., Manka, J. T., Rodriguez, A. L., Vinson, P. N., Niswender, C. M., Weaver, C. D., Jones, C. K., Conn, P. J., Lindsley, C. W., Stauffer, S. R. 2011. Synthesis and SAR of centrally active mGlu5 positive allosteric modulators based on an aryl acetylenic bicyclic lactam scaffold. Bioorg. Med. Chem. Lett. 21, 1350-1353.




# 5 Anti-absence activity of mGlu1 and mGlu5 receptor enhancers and their interaction with a GABA reuptake inhibitor: effect of local infusions in the somatosensory cortex and thalamus

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# Summary

**Objective:** Glutamate and GABA are the key neurotransmitter systems in the cortico-thalamo-cortical network, involved in normal and pathological oscillations such as spike-wave discharges (SWDs) characterizing different forms of absence epilepsy. Metabotropic glutamate (mGlu) and GABA receptors are widely expressed within this network. Here, we examined the effects of two selective positive allosteric modulators (PAMs) of mGlu1 and mGlu5 receptors, the GABA reuptake inhibitor, tiagabine, and their interaction in the somatosensory cortex and thalamus on SWDs in WAG/Rij rats.

**Methods:** Male WAG/Rij rats were equipped with bilateral cannulas in the somatosensory cortex (S1po) or the ventral basal complex of the thalamus (VB), and with cortical EEG electrodes. Rats received a single dose of the mGlu1 receptor PAM, RO0711401, or the mGlu5 receptor PAM, VU0360172, various doses of tiagabine, or VU0360172 combined with tiagabine.

**Results:** Both PAMs suppressed SWDs regardless of the site of injection. Tiagabine enhanced SWDs when injected in the thalamus, but, unexpectedly, suppressed SWDs in a dose-dependent manner when injected in the cortex. Intracortical co-injection of VU0360172 and tiagabine produced slightly larger effects as compared to either VU0360172 or tiagabine alone. Intrathalamic co-injections of VU0360172 and sub-threshold doses of tiagabine caused an anti-absence effect similar to that exhibited by VU0360172 alone in the first 10 min. At 30 min, however, the anti-absence-effect of VU0360172 was prevented by sub-threshold doses of tiagabine, and the combination produced a paradoxical pro-absence effect at 40 and 50 min.

**Significance:** These data (i) show that mGlu1 and mGlu5 receptor PAMs reduce absence seizures acting at both thalamic and cortical levels; (ii) demonstrate for the first time that tiagabine, in spite of its established absence-enhancing effect, reduces SWDs when injected in the somatosensory cortex; (iii) indicate that the efficacy of VU0360172 in the thalamus may be critically affected by the availability of (extra)synaptic GABA.

**Key words**: Glutamate, GABA; Absence Epilepsy; WAG/Rij rats; mGlu PAM

# Introduction

Dysfunction in either glutamatergic or GABAergic neurotrasmission is known to be one of the causes responsible for the initiation and spread of seizures, including absence epilepsy. Known as the electroencephalographic hallmark of absence seizures, the archetypical spike-wave discharges (SWDs) are initiated in the deep layers of the somatosensory cortex and quickly spread to the cortico-thalamo-cortical (C-T-C) network.[1,2] This network consists of glutamatergic projections from the deep layer cortical neurons to ventrobasal (VB) thalamic nuclei including the PO, and to the reticular thalamic nucleus (nRT); glutamatergic projections from VB thalamic nuclei to the cortex and nRT; and GABAergic projection from the nRT to VB thalami nuclei.[2,3] Several mGlu receptor subtypes appear strategically distributed at the synapses of the C-T-C loop. In the cortex, group-I mGlu receptors (namely mGlu1 and mGlu5 receptors) are expressed post-synaptically on GABAergic interneurons.[4,5,6] At thalamic level, group I mGlu receptors are present post-synaptically on glutamatergic neurons of the VB complex reviewed by Ngomba et al., 2011.[7] These neurons express moderate levels of mGlu5 receptor immunoreactivity,[8] while the mGlu1 receptors mainly reside in the perisynaptic area.[9,10,11,12] Modulatory effects of individual mGlu receptor subtypes on both excitatory and inhibitory synaptic transmissions in the C-T-C circuit have been found, and subtype-selective mGlu receptor ligands were proposed as potential candidates for novel antiabsence drugs.[7]

Studies with mGlu receptor ligands have been conducted in WAG/Rij rats, which develop spontaneous absence seizures after 2-3 months of age.[7] Systemic administration of compounds RO0711401 and VU0360172, which behave as selective positive allosteric modulators (PAMs) of mGlu1 and mGlu5 receptors, respectively, decrease the incidence of SWDs in symptomatic WAG/Rij rats.[13,14] No tolerance develops to the anti-absence activity of VU0360172, whereas the activity of RO0711401 declines after the first three days of repeated administrations.[15] Where precisely within the C-T-C circuit activation of mGlu1 or mGlu5 receptors reduces SWDs is unknown. Here, we have addressed this question by locally injecting either VU0360172 or RO0711401 in the VB part of the thalamus or in the somatosensory cortex.

Next to the role of glutamate, GABA also holds a key position in the control of SWDs. It is well known that systemically administered GABA-mimetics, such as tiagabine, acting by blocking the reuptake of GABA via the high affinity GABA transporter, GAT-1,[16] aggravate the incidence of SWDs.[17] Here, we also investigated whether GABAergic regulation of absence seizures differs in the thalamus and cerebral cortex. WAG/Rij rats represent an appropriate model to examine this issue because in these rats as well as in GAERS (genetic absence epileptic rats of Strasbourg, [18] a high excitability of S1po is a prerequisite for the initiation of SWDs [1,2] that are then sustained by an enhanced tonic GABAergic inhibition in the thalamus.[19,20] Finally, using tiagabine, we examined whether, and in which direction, an increased availability of (extra)synaptic GABA influences responses to the mGlu5 receptor PAM, VU0360172, in the thalamus and somatosensory cortex.

# Materials and Methods

## Animals

One hundred and thirty one male WAG/Rij rats were used for all experiments. Of these, eight animals were excluded because of a wrong position of the cannulas either in the cortex or in the thalamus. All rats were born and raised at Radboud University Nijmegen, The Netherlands, and had a mean body weight of about 350 g at 9 months of age. Rats of this age have about 16–20 SWDs per hour, adding several hundred SWDs per day.[21] The animals were housed in pairs in Macrolon cages, kept under controlled conditions (20°C, 60% humidity) in a room with a reversed light–dark cycle (white light on from 9 p.m. to 9 a.m.), with food and drinking water always available. After surgery, rats were kept individually. Animals were handled regularly before starting EEG registrations to reduce handling stress imposed by the local injections. The study was performed in accordance with the guidelines of the European Community for the use of experimental animals and was approved by local ethics committee for animal studies (RU-DEC). All efforts were made to reduce discomfort experienced by the animals and to keep the number of animals as low as possible.

## Drugs and experimental protocol

VU0360172 (N-clyclobutyl-6-[2-3(fluorophenyl) ethynyl] pyridine-3-carboxamine), a selective mGlu5 receptor PAM, was obtained from Vanderbilt University Medical Center.[22] RO0711401 (9H-xanthene-9-carboxylic acid (4-trifluoromethyl-oxazol-2-yl) amide, a selective mGlu1 receptor PAM, was kindly provided by Hoffmann-La Roche (Basel, Switzerland). Tiagabine (Hydrochloride, monohydrate), a GABA-reuptake inhibitor, was purchased from Siegfried Chemie AG.

In all 3 experiments (Table 1), bilateral microinfusions in the peri-oral region of the somatosensory cortex (S1po) or in the VB thalamic nuclei were performed using artificial cerebro spinal fluid (ACSF) as vehicle. Drugs were soluble in ACSF at concentrations of 1 mg/ml (VU0360172, RO0711401) or 2 mg/ml (tiagabine). In experiments with single injections, drugs were always microinfused in a volume of 1 µl. Doses were 1 µg for VU0360172 and RO0711401, and 0.5, 1 or 2 µg for tiagabine. In experiments in which VU0360172 and tiagabine were co-administered, the two drugs were infused with a 3 min interval. In the S1po, both VU0360172 and tiagabine were infused at doses of 1 µg in a volume of 1 µl (i.e., 1 µl + 1 µl with 3 min of interval). In the thalamus, VU0360172 was infused at the dose of 1 µg whereas tiagabine was infused at the dose of 0.5 µg (always in a volume of 1 µl). ACSF was infused twice with 3 min interval in control animals. Infusions were performed by means of a Hamilton syringe at a flow rate of 1 µl per min and the injection needle remained in the cannula after each single injection for 2 min to prevent backflow. The needle necessary for doing local injections did not protrude from the guide cannulas in order to avoid a spreading depression induced by the local injection.[23]

**Table 1** Overview of the three local injection experiments with drugs, doses, and side of drug application.

| | Drug | Dose | Brain region |
|---|---|---|---|
| Experiment 1 | VU0360172 | 1 μg (n = 8 and n = 9) | SIPo and VB |
| | RO0711401 | 1 μg (n = 8 and n = 8) | SIPo and VB |
| | ACSF | (n = 9 and n = 9) | SIPo and VB |
| Experiment 2 | Tiagabine | 1 μg (n = 8)<br>2 μg (n = 8) | SIPo |
| | Tiagabine | 0.5 μg (n = 4)<br>1 μg (n = 5)<br>2 μg/μl (n = 5) | VB |
| | ACSF | (n = 8 and n = 8) | SIPo and VB |
| Experiment 3 | VU0360172 followed by tiagabine (3 min interval) | 1 μg + 1 μg (n = 8) | SIPo |
| | VU0360172+ followed by tiagabine (3 min interval) | 1 μg + 0.5 μg (n = 9) | VB |
| | ACSF followed by ACSF (3 min interval) | (n = 8) | SIPo |
| | ACSF followed by ACSF (3 min interval) | (n = 9) | VB |

The injection volume was always 1 μl for each drug or ACSF injection.

In all experiments, cortical EEG was recorded and motor behavior was quantified as detailed in the Supporting Information. Surgical procedures, coordinates used for the implantation of guide cannulas, and the procedure we have used to determine the position of the guide cannulas are described in detail in the Supporting Information.

## Statistical analysis

The effects of intracortical or intrathalamic drug injections on the incidence and mean duration of SWDs, and locomotor activity were tested in separate repeated-measures ANOVAs with incidence and mean duration of SWDs or amplitude of the PIR as dependent variables. The hourly incidence (number) and mean of SWDs in the 2 hours before injection were determined and differences between groups were analyses with a simple one-factor ANOVA (groups) for all three experiments. For all post-injection analyses, the time of EEG recording (6x10 minutes blocks post injection) was used as the within subjects factor, group (either site of injection of different drugs) was used as between subjects factor. In Experiment 2 and 3 repeated measures were conducted as well, but now

different doses of tiagabine in cortex and thalamus (Experiment 2), or the different groups (Experiment 3 co-injection group, ACSF + ACSF), were used as between subjects factors, all followed by post-hoc tests, if appropriate. One way ANOVAs and Duncan post-hoc tests were used to isolate differences between the locations, doses or different drug treated groups at the different 10 minute episodes. Results are expressed as mean ± SEM and calculations were obtained using SPSS 19 software. The level of statistical significance was set at $p < .05$.

# Results

## Effects of microinfusion of VU0360172, RO0711401 or ACSF in the S1po cortex and ventrobasal thalamus on absence seizures in WAG/Rij rats

EEG was recorded in symptomatic WAG/Rij rats at baseline and following bilateral microinfusion of VU0360172 (1 µg/µl), RO0711401 (1 µg/µl) or vehicle (ACSF, 1 µl) in the S1po region of the somatosensory cortex or in the VB thalamus. A representative trace of a typical SWDs recorded by EEG is shown in Fig. 1A. No significant differences were found in the two hours preceding drug injections among the 6 groups of animals receiving each of the two drugs or vehicle in either the S1po or the VB thalamus with respect to the incidence of SWDs ($F=2.048$, df 5,45, $p =.086$, $\eta^2=.16$), mean duration of SWDs ($F=0.460$, df 5,45, $p =.804$, $\eta^2=.04$), and locomotor activity (measured as the amplitude of the PIR) ($F=1.607$, df 5,45, $p =.174$, $\eta^2=.13$).

EEG was recorded for two more hours post drug or vehicle injections in the S1po or the VB thalamus, and the incidence and mean duration of SWDs was measured in blocks of 10 min. Two-way ANOVA for repeated measures applied to the analysis of the incidence of SWDs in blocks of 10 min after bilateral injections of VU0360172 (n = 8), RO0711401 (n = 8), or vehicle (n = 9) in the S1po showed a time effect ($F= 69.25$, df 5,155, $p< .001$, $\eta^2= .691$), a drug effect ($F=29.56$, df 2, 31, $p < .001$, $\eta^2=.656$) and a significant interaction between time and drugs ($F=10.28$, df 10,155 $p <.001$, $\eta^2=.399$). Post-hoc tests revealed that both VU0360172 and RO0711401 reduced SWD incidence in the first 40 min post-injection with no difference between the two drugs at all time-points (Figure 1B).

The same analysis on SWD incidence after injections of VU0360172 (n = 9), RO0711401 (n = 8) or vehicle (n = 9) in the VB thalamus revealed a time effect ($F=39.48$, df 5,224, $p< .001$, $\eta^2= .552$), a drug effect ($F=44.61$, df 2,32, $p< .001$, $\eta^2=.736$), and a significant interaction effect between time and drugs ($F=5.48$ df 14,224 $p< .001$, $\eta^2=.255$), (Figure 1C). Post-hoc analyses across the six 10-min blocks showed varying differences across time. VU0360172 was more ($p<.001$) effective than RO0711401 in suppressing the incidence of SWD in the first 10 min post-injections, with both drugs showing significant effects as compared to the ACSF control group. Between 10-20 and 20-30 min post-injections, RO0711401 and

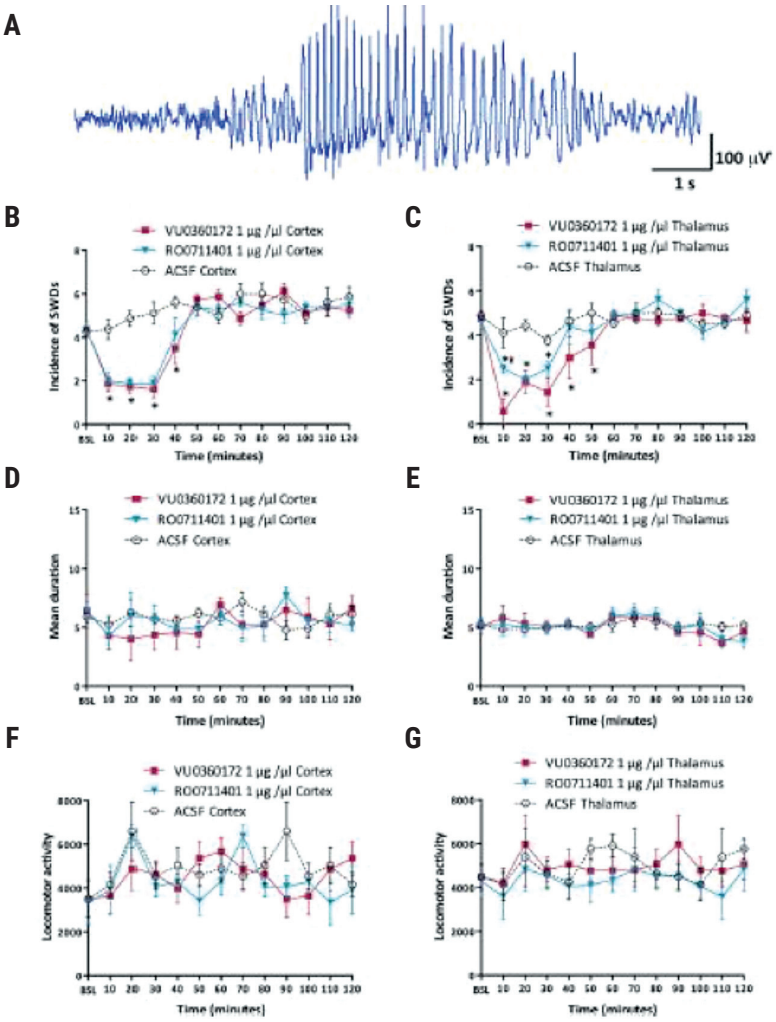


**Figure 1** Pharmacologic enhancement of mGlu or mGlu5 receptors in the somatosensory (S1po) cortex or ventrobasal (VB) thalamus inhibits absence seizures in WAG/Rij rats. A representative EEG recording of a typical SWD episode is shown in (A). The incidence of SWDs (in blocks of 10 min) in WAG/Rij rats locally infused in the somatosensory (S1po) cortex with VU0360172 (1 µg; n = 8), RO071140 (1 µg; n = 8), or ACSF (n = 9) is shown in (B). The incidence of SWDs after infusion of VU0360172 (1 µg; n = 9), RO071140 (1 µg; n = 8), or ACSF (n = 9) in the VB thalamus is shown in (C). The injection volume was always 1 µl. Values are means + SEM. $p < 0.05$ (two-way ANOVA + Duncan's t-test) compared to the corresponding ACSF values (*), or the corresponding VU0360172 values (#). The corresponding values of mean duration of SWDs and locomotor activity (means + SEM) are shown in (D, E) and (F, G), respectively.

VU0360172 were equally effective in suppressing SWDs. Between 30 and 50 min post injection RO0711401 lost its activity whereas VU0360172 still significantly reduced the incidence of SWDs.

Differences in the efficacy of VU0360172 injected in the cortex and thalamus were found only after 50 min post-injection ($F=14,205$, df 2,32, $p < .001$, $\eta^2=.470$), when the SWD-suppressing effects of VU0360172 were larger in the thalamus. This difference was no longer observed at 60 min post-injection. No statistical difference was found between intrathalamic and intracortical injection of RO0711401 with respect to the incidence of SWD.

None of the treatments caused significant differences in the mean duration of SWDs (Fig. 1D,E) and in locomotor activity (Fig. 1F,G).

## Intracortical and intrathalamic injections of tiagabine produced opposite effects on absence seizures in WAG/Rij rats

Tiagabine was injected at doses of 1 or 2 µg in the S1po (n = 8 in both groups), and 0.5, 1, or 2 µg in the VB thalamus (n = 4, 5, and 5, respectively). Control rats received ACSF (n = 8 in both S1po and thalamus). There were no differences between the groups before drug administration. Intracortical injection of tiagabine reduced the incidence of absence seizures. Two-way ANOVA for repeated measures applied to the analysis of the incidence of SWDs after cortical injections of tiagabine or vehicle showed a time effect ($F=107.93$, df 5,105, $p< .001$, $\eta^2=.837$), with a large reduction of SWDs in the first 40 minutes post injection (Fig. 2A). There was also a dose effect ($F=164.69$, df 2, 21, $p< .001$, $\eta^2=.940$), and an interaction between time and dose ($F=42.77$, df 10,105, $p< .001$, $\eta^2=.803$). Post-hoc analyses showed various differences across the six 10-min blocks: Between 10-40 minutes post-injection, the high dose of tiagabine was more effective in suppressing SWDs compared to the low dose of tiagabine and to ACSF. The low dose also suppressed SWDs during the first 30 min, as compared to ACSF. The low and high doses of tiagabine lost their anti-absence effect at 40 and 50 min post-injections, respectively.

The increase in the incidence of SWDs was obtained after intrathalamic injection of tiagabine. Two-way ANOVA for repeated measures showed a time effect ($F= 73.643$, df 5, 80, $p< .001$, $\eta^2= .822$), with an increase in the incidence of SWDs being present in the first 40 minutes after injection of tiagabine (Fig. 2B). There was also a dose effect ($F= 656.218$, df 3, 16, $p< .001$, $\eta^2=.992$) and an interaction between time and dose ($F=45.84$, df 15, 80 $p< .001$, $\eta^2=.896$). Post-hoc analysis across the six 10-min blocks showed that, between 10 and 30 min post-injection, the highest dose of tiagabine (2 µg) increased the incidence of SWDs to a greater extent than the mid dose (1 µg). The lowest dose of tiagabine (0.5 µg) was inactive. At 40 min post injection, only the highest dose of tiagabine was still effective in reducing the incidence of SWDs.

Treatment with tiagabine did neither change the mean duration of SWDs (Fig. 2C,D) nor locomotor activity scores (Fig. 2E,F).

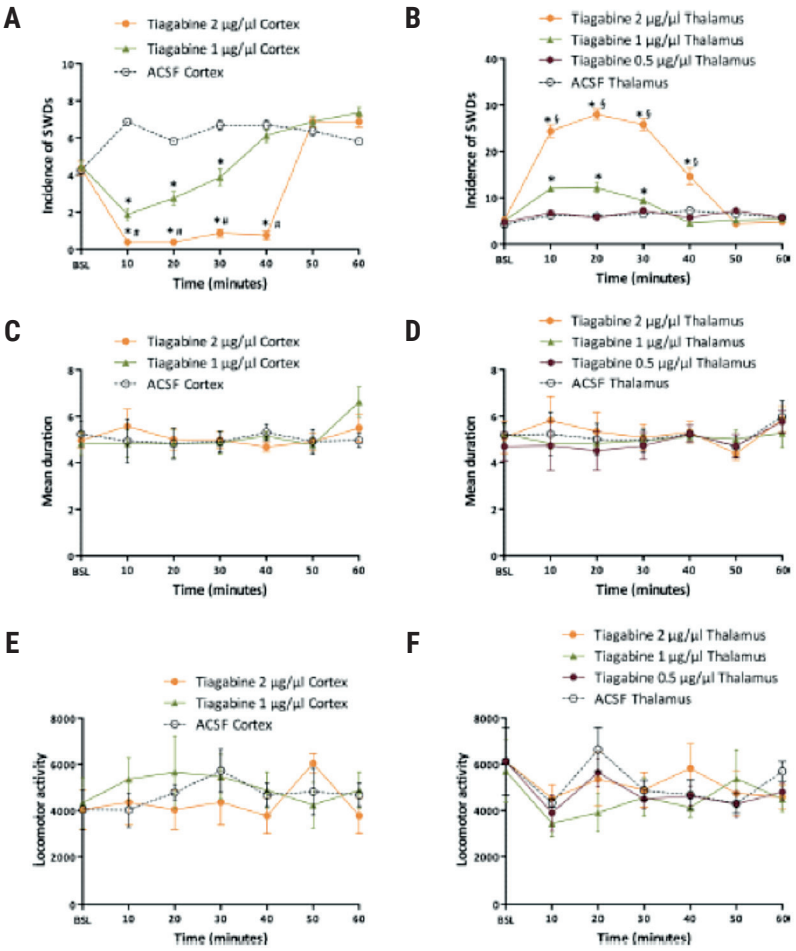


**Figure 2** Effect of tiagabine locally infused in the somatosensory (S1po) cortex or ventrobasal (VB) thalamus on absence seizures in WAG/Rij rats. The effects of two doses of tiagabine (1 µg; n = 8; or 2 µg; n = 8) or ACSF (n = 8) infused in the S1po cortex, and the effects of three doses of tiagabine (0.5 µg/µl; n = 4; 1 µg/µl; n = 5; 2 µg/µl; n = 5) or ACSF (1 µl; n = 8) infused in the VB thalamus are shown in (A) and (B), respectively. The injection volume was always 1 µl. Values are means + SEM. $p < 0.05$ (two-way ANOVA + Duncan's t-test) compared to the corresponding ACSF values (*), to the corresponding values obtained with 1 lg tiagabine (#) in (A), or to the corresponding values obtained with 0.5 µg tiagabine (§) in (B). In (B), the same symbol (either * or §) is referred to the overlapping values obtained inrats treated with 1 or 2 lg of tiagabine. The corresponding values of mean duration of SWDs and locomotor activity (means + SEM) are shown in (C,D) and (E, F), respectively.

### Effects of combined injections of VU0360172 and tiagabine in the cortex and thalamus

In all experiments, injection of VU0360172 (1 µg in both S1po and VB thalamus) preceded by 3 min the injection of tiagabine (1 µg in the S1po, and 0.5 µg in the VB thalamus; n = 8 and 9, respectively). Control rats received two sequential injections of ACSF in the S1po or in the VB thalamus (n = 8 and 9, respectively). There were no differences between the groups before drug administration. Data obtained after intracortical injections are shown in Fig. 3A. Two-way ANOVA for repeated measures after injections of VU0360172 + tiagabine and ACSF + ACSF showed a time ($F= 14.132$, df 5,70, $p< .001$, $\eta^2= .502$), and a group effect ($F= 102.86$, df 1,14, $p< .001$, $\eta^2= .880$). In the first 10-40 min post injection, the combination of VU0360172 and tiagabine substantially reduced the incidence of SWDs with respect to the control group. In the first 20 min post-injection the reduction in the incidence of SWDs was greater than that previously observed with VU0360172 alone (-67% and -72% with VU0360172 plus tiagabine at 10 and 20 min post-injection, respectively, in Fig. 3A vs. -57% and -60% with VU0360172 alone at 10 and 20 min post-injection, respectively, in Fig. 1B). At 50 and 60 min there was a slight reduction in the incidence of SWDs, with no significant difference from the control group.

Data obtained after intrathalamic injections of VU0360172 (1 µg/µl) and tiagabine (0.5 µg/µl) are shown in Fig. 3B. Here, there was also a time effect (blocks) ($F= 5.23$, df 5,80, $p< .001$, $\eta^2= .231$), and an interaction between time and group ($F=2.90$, df 5,80 $p<.001$, $\eta^2=.154$). Post-hoc analysis across the six 10-min blocks showed that in the first 10 min post-injection, the combination between VU0360172 and tiagabine reduced the incidence of SWDs compared to 2 x ACSF. Combination of the two drugs was ineffective at 20 min and caused a significant increase in the incidence of SWDs at 30 and 40 min post injection, again in comparison with 2 x ACSF.

None of these treatments had any effect on the mean duration of SWDs (Fig. 3C,D) and locomotor activity (Fig. 3E,F).

## Discussion

Our experiments were a follow-up from the evidence that systemic administration of group-I mGlu receptor PAMs reduces the incidence of SWDs in WAG/Rij rats.[7,13,14] Here, we showed that this effect could be induced by injecting mGlu1 or mGlu5 receptor PAMs in both the S1po and ventrobasal thalamic nuclei. There was a difference in the thalamic and cortical response to RO0711401 and VU0360172. The two drugs were equally effective in reducing SWDs when injected in the cortex; in contrast, the mGlu5 PAM, VU036012, displayed a greater efficacy than the mGlu1 PAM, RO0711401, when injected in the thalamus. The somatosensory cortex is the main site of origin of SWDs in WAG/Rij and GAERS rats,1 and this region is highly excitable. [2] In the somatosensory cortex, mGlu1α

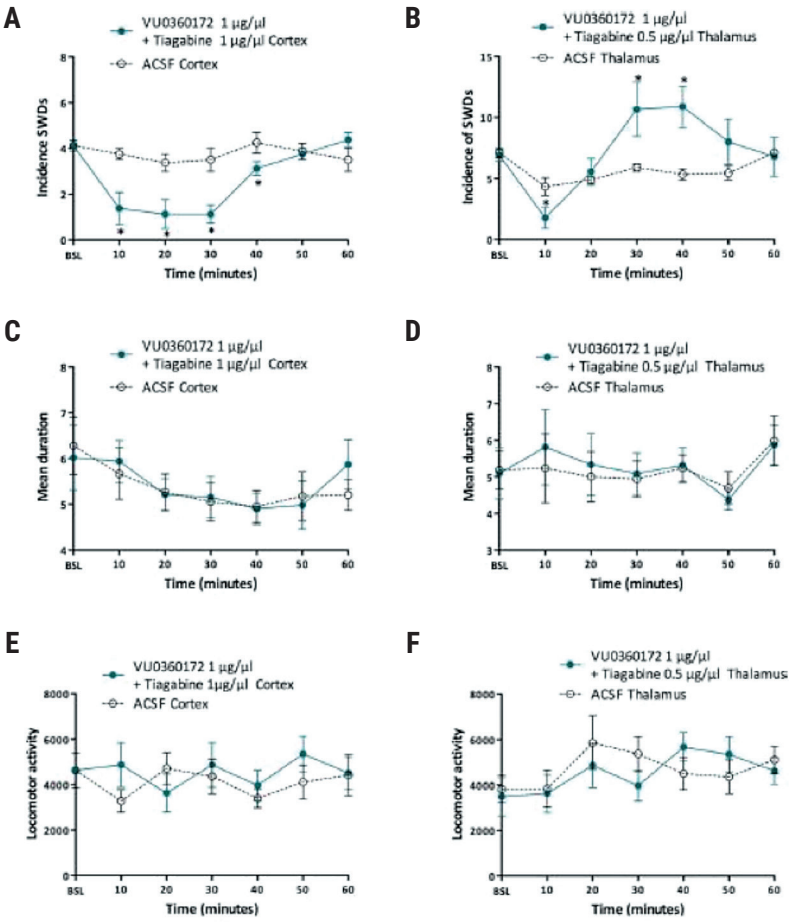


**Figure 3** Combined effect of VU0360172 and tiagabine on the incidence of absence seizures in WAG/Rij rats. The effects of intracortical injections of VU0360172 (1 µg) plus tiagabine (1 µg) (n = 8) or ACSF + ACSF (n = 8) on the incidence of SWDs are shown in (A); the effects of intrathalamic injections of VU0360172 (1 lg) plus tiagabine (0.5 µg) (n = 9) or ACSF + ACSF (n = 9) are shown in (B). Values are means + SEM. Values are means + SEM. *p < 0.05 (two-way ANOVA + Duncan's t-test) versus the corresponding ACSF values. The corresponding values of mean duration of SWDs and locomotor activity (means + SEM) are shown in (C, D) and (E, F), respectively. VU0360172 and tiagabine were microinfused with 3 min of interval. Control rats received two injections of ACSF with 3 min of interval.

receptors are expressed by somatostatin-positive, calretinin-positive, and calbindin-positive, but not by fast-spiking parvalbumin-positive, GABAergic interneurons.[4,5,6] mGlu5 receptors are found in both somatostatin-positive and parvalbumin-positive GABAergic interneurons,[5,6] as well as in pyramidal neurons.[24] Because the efficacy of intracortically injected VU0360172 and RO0711401 was identical, it is possible that a cell type expressing both mGlu1 and mGlu5 receptors is a common target for the two PAMs. We hypothesize therefore that activation of either mGlu1 or mGlu5 receptors expressed by somatostatin-sensitive and other types of regular spiking GABAergic interneurons suppresses SWDs by enhancing GABAergic inhibition onto pyramidal neurons. This hypothesis is consistent with the finding that intracortical injection of tiagabine, which inhibits the high affinity GABA transporter, GAT-1, reduced SWDs. This leaves us with the suggestion that enhancing mGlu1/5-mediated activation of cortical GABAergic interneurons or inhibiting GABA reuptake would produce the same effect. Hence, it is not surprising that intracortical injection of VU0360172 plus tiagabine reduced SWDs to a slightly larger extent than injection of VU0360172 alone.

Both mGlu1 and mGlu5 receptors are present postsynaptically on VB thalamic neurons, and only mGlu5 receptors are found at moderate levels in GABAergic neurons of the reticular thalamic nuclei (reviewed by Ngomba et al., 2011)7. Both mGlu1 and Glu5 receptors are coupled to Gq/11, and their activation stimulates phospholipase-Cβ4(PLC-β) with ensuing hydrolysis of phosphatidylinositol-4,5-bisphosphate and formation of inositol-1,4,5-trisphosphate and diacylglycerol (reviewed by Nicoletti et al., 2011). [25] Mutations of PLC-β4 at thalamic level have been shown to enhance bursting of thalamo-cortical projection neurons in a T-type $Ca^{2+}$ channel-dependent fashion, resulting in absence epilepsy.[26] Thus, activation of either mGlu1 or mGlu5 receptors present in VB thalamic neurons might restrain the occurrence of absence seizures by negatively regulating the activity of T-type $Ca^{2+}$ channels on thalamo-cortical cells. However, intrathalamic injection of VU036172 caused a more robust and prolonged suppression of SWDs as compared to intrathalamic injection of RO0711401, suggesting that an additional mechanism that is peculiar to mGlu5 receptors contributes to restrain the occurrence of SWDs. A major difference between mGlu1 and mGlu5 receptors is that only the latter are found on astrocytes, both in the thalamus and cerebral cortex.[27,28] Astrocytes are key players in the regulation of thalamic oscillations because they clear extracellular glutamate and GABA, thereby limiting the activation of extrasynaptic glutamate and GABA receptors.

An increased inhibitory GABAergic transmission at the synapses between reticular thalamic neurons and VB thalamic neurons sustains the occurrence of SWDs (reviewed by Blumenfeld, 2005)[2], and this explains the pro-absence effect seen after systemic administration of drugs that enhance GABAergic transmission, such as tiagabine, vigabatrin, and the neurosteroid allopregnanolone in both GAERS and WAG/Rij rats.[16,29,18,30] Intrathalamic injections of the GABA-transaminase inhibitor, γ-vinyl GABA, the GABAA receptor agonist, muscimol, or the neurosteroids, allopregnanolone or ganaxolone also

increase the incidence of SWDs.[31,32] Tiagabine enhances tonic inhibition of thalamic relay neurons by inhibiting GABA re-uptake, and, therefore, facilitating the endogenous activation of extrasynaptic GABA receptors.[33,19,34] Hence, the enhanced incidence of SWDs found after intrathalamic injection of tiagabine was fully expected and suggests that the pro-absence activity in the thalamus prevails over the anti-absence activity in the cortex after systemic injection of tiagabine.

Experiments in which the mGlu5 receptor PAM, VU036172 was co-injected with sub-threshold doses of tiagabine in the VB thalamus produced interesting results. If combined with tiagabine, VU036172 was still effective in reducing the incidence of SWDs in the early post-injection time (10 min), but then lost this effect at 20 min, and displayed a paradoxical pro-absence effect at 30 and 40 min. This suggests that the regulation of thalamic oscillations by mGlu5 receptors is shaped by extracellular GABA, or, alternatively, that the mGlu5 receptor controls the activity of the GABA transporter, GAT-1, in astrocytes or in GABAergic fibers afferent to VB thalamic nuclei. Activation of glial mGlu5 receptors is known to regulate the expression of the glial glutamate transporters, GLT-1 and GLAST.[35] If this regulation extends to GAT-1, we may suggest the following scenario. Pharmacological enhancement of mGlu5 receptors produces pleiotropic effects in the VB thalamus, including an enhanced clearance of extracellular GABA, which might be critical for the suppression of SWDs. In the presence of a GAT-1 inhibitor (e.g., tiagabine) activation of mGlu5 receptors will not stimulate GABA reuptake, and the progressive increase in extracellular GABA will block the anti-absence action mediated by mGlu5 receptor stimulation. Studies on GABA uptake in cultured astrocytes or studies on tonic inhibition in thalamic slices in epileptic and non-epileptic rats are needed to verify this hypothesis.

In conclusion, group I metabotropic receptors are involved in the control of absence seizures through the entire C-T-C network that is responsible for the pathological oscillations associated with SWDs. However, the consequences of stimulation are site dependent: stimulating group I mGlu receptors in the cortex may enhance GABA-ergic inhibition onto pyramidal neurons and reduces SWDs. Stimulation of group I mGlu receptors in thalamic relay neurons might reduce tonic inhibition thereby reducing SWDs. In the presence of tiagabine, activation of thalamic mGlu5 receptors will not stimulate GABA reuptake, and the progressive increase in extracellular GABA will abolish the anti-absence effect mediated by mGlu5 receptor stimulation. A schematic diagram illustrating our hypothesis on the regulation of absence seizures by cortical and thalamic group-I mGlu receptors and the potential interaction with GABAergic transmission is shown in Fig. 4.

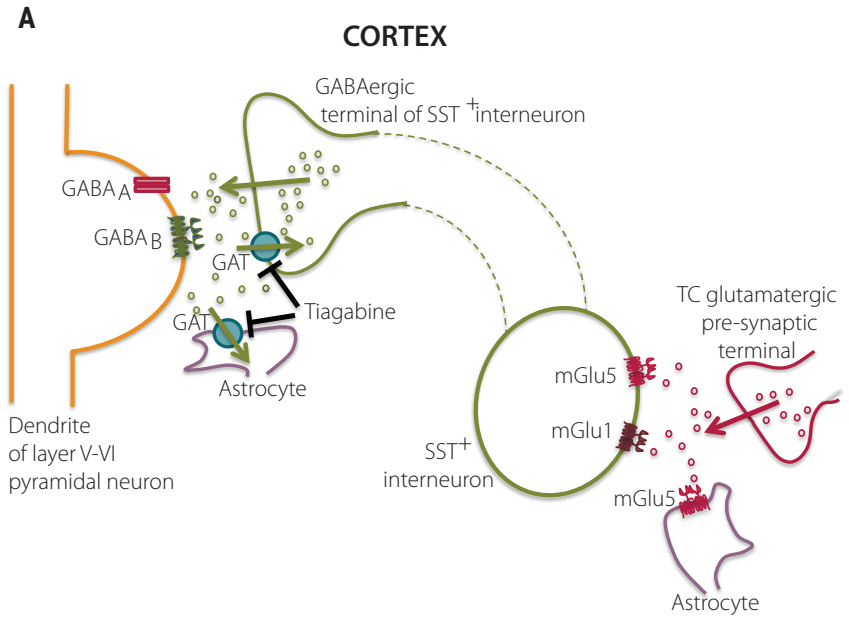


**Figure 4** Mechanistic hypothesis of the role played by cortical (A) or thalamic (B) mGlu1 and mGlu5 receptors in the modulation of absence seizures. The diffusion of either GABA (rounded dots and arrows in green) or glutamate (rounded dots and arrows in red) within and out of the synaptic terminals and glial cells are depicted. In the S1po cortex, pharmacologic enhancement of mGlu1 or mGlu5 receptors might reduce absence seizures by activating somatostatin (SST)–positive GABAergic interneurons, and tiagabine might produce the same effect by enhancing GABA levels at the synapses between SST+ interneurons and pyramidal neurons. In the ventrobasal thalamus, tiagabine enhances absence seizures by increasing synaptic and extrasynaptic GABA levels, thereby facilitating the activity of T-type voltage-sensitive $Ca^{2+}$ channels. mGlu1 and mGlu5 receptors might protect against absence seizures by directing modulating T-type $Ca^{2+}$ channels (modulation of T-type channels by phospholipase C has been reported[26]) or through a blockade of the glial glutamate transporter.[35] Tiagabine might act via blockade of the presynaptic and glial GABA transporter. Data on the interaction between VU0360172 and tiagabine raise the possibility that mGlu5 receptors regulate the expression or activity of GAT-1 in GABAergic terminals and/or astrocytes.

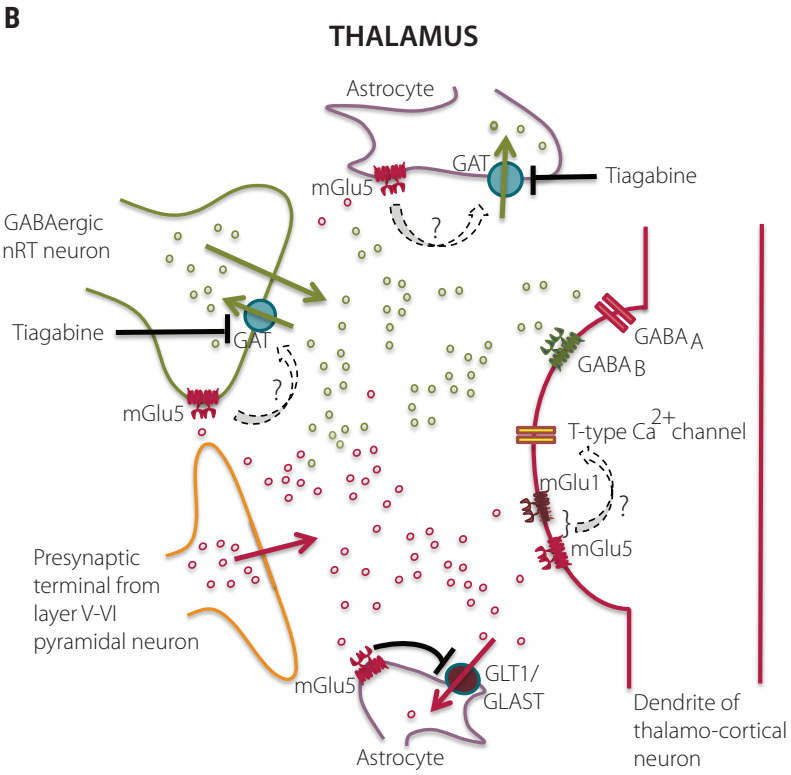


**Figure 4** Continued.

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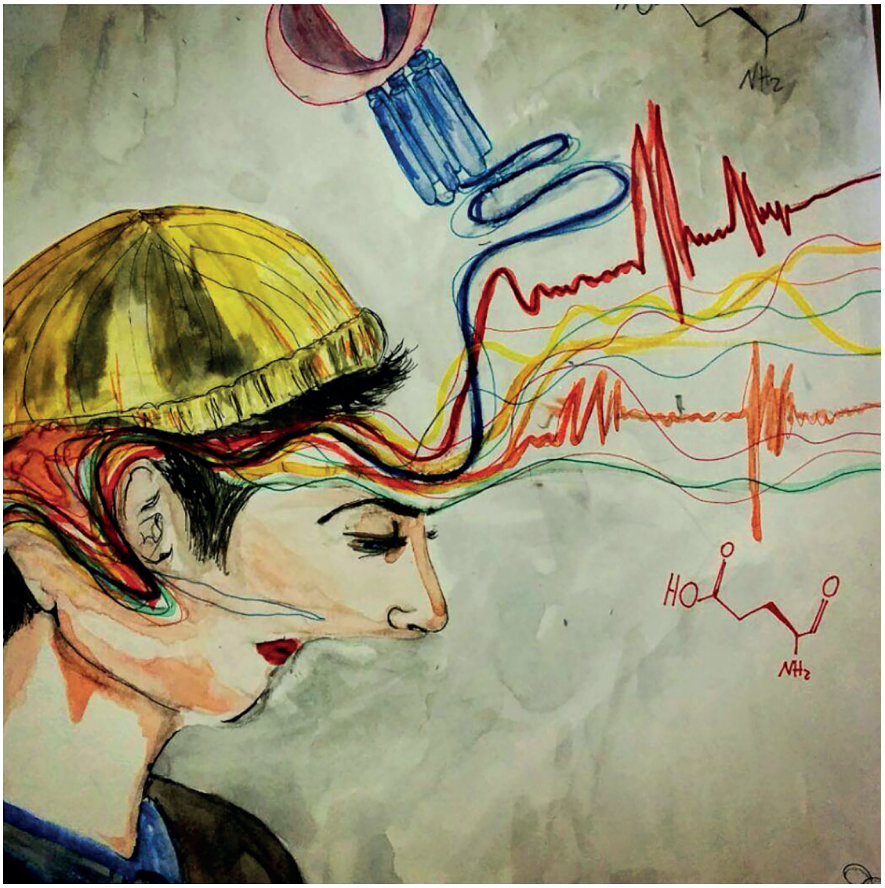
## Disclosure statement

Authors declare that there are no conflicts of interest to be disclosed. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

# References


1. Meeren HK, Pijn JP, van Luijtelaar EL, et al. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci. 2002 Feb 15;22:1480-95.
2 Blumenfeld H. Cellular and network mechanisms of spike-wave seizures. Epilepsia 2005;46:21-33.
3. van Luijtelaar G, Sitnikova E. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 2006;30:983-1003.
4. Stinehelfer S, Vruwink M, Burette A. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 2000 Apr 7;861:37-44.
5. Kerner JA, Standaert DG, Penney JB Jr, et al. Expression of group one metabotropic glutamate receptor subunit mRNAs in neurochemically identified neurons in the rat neostriatum, neocortex, and hippocampus. Brain Res Mol Brain Res. 1997 Sep;48:259-69.
6. Sun QQ, Zhang Z, Jiao Y, et al. Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. J Neurophysiol. 2009 May;101:2679-92.
7. Ngomba RT, Santolini I, Salt TE, Ferraguti F, Battaglia G, Nicoletti F, van Luijtelaar G. Metabotropic glutamate receptors in the thalamocortical network: strategic targets for the treatment of absence epilepsy. Epilepsia 2011;52:1211–22.
8. Romano C, Sesma MA, McDonald CT, et al. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J Comp Neurol.1995;355:455–469.
9. Baude A, Nusser Z, Roberts JD, et al. The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 1993;11:771–87.
10. Molnár E, McIlhinney RA, Baude A, et al. Membrane topology of the GluR1 glutamate receptor subunit: epitope mapping by site-directed antipeptide antibodies. J Neurochem1994; 63:683–93.
11. Lujan R, Nusser Z, Roberts JD, et al. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci. 1996;8:1488–500.
12. Mateos JM, Benítez R, Elezgarai I, et al. Immunolocalization of the mGluR1b splice variant of the metabotropic glutamate receptor 1 at parallel fiber-Purkinje cell synapses in the rat cerebellar cortex. J Neurochem. 2000;74:1301–9.
13. Santolini I, Biagioni F, et al. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology 2011;60:1281–1291.
14. D'Amore V, Santolini I, van Rijn CM, et al. Potentiation of mGlu5 receptors with novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacology 2013;66:330–338.
15. D'Amore V, Santolini I, Celli R, et al. Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats. Neuropharmacology 2014;85:91–103.
16. Pow DV, Sullivan RK, Williams SM, et al. Differential expression of the GABA transporters GAT-1 and GAT-3 in brains of rats, cats, monkeys and humans. Cell Tissue Res.2005;320:379–92.
17. Coenen AM, Blezer EH, van Luijtelaar EL. Effects of the GABAuptake inhibitor tiagabine on electroencephalogram, spike-wave discharges and behaviour of rats. Epilepsy Res.1995;21:89–94.
18. Depaulis A, van Luijtelaar G. Genetic models of absence epilepsy in the rat. In: Pitkanen A, Schwartzkroin P, Moshe S (Ed.), Elsevier Inc, San Diego, CA. Models of Seizures and Epilepsy 2006:233–248.
19. Cope DW, Di Giovanni G, Fyson SJ, et al. Enhanced tonic GABA inhibition in typical absence epilepsy. Nat Med.2009;15:1392–1398.
20. Govindaiah G, Cox CL. Metabotropic glutamate receptors differentially regulate GABAergic inhibition in thalamus. J Neurosci.2006;26:13443–53.
21. van Luijtelaar ELJM, Coenen AML. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett.1986;70:393–397.
22. Williams R, Manka JT, Rodriguez AL, et al. Synthesis and SAR of centrally active mGlu5 positive allosteric modulators based on an aryl acetylenic bicyclic lactam scaffold. Bioorg Med Chem Lett.2011;21:1350–1353.
23. Samotaeva IS, Tillmanns N, van Luijtelaar ELJM, et al. Intracortical Microinjections may cause Spreading Depression and suppress Absence Seizures. Neuroscience 2013;230:50–55.

24. Wijetunge LS, Till SM, Gillingwater TH, et al. mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. J Neurosci. 2008;28:13028–37.
25. Nicoletti F, Bockaert J, Collingridge GL, et al. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 2011;60:1017–41.
26. Cheong E, Zheng Y, Lee K, et al. Deletion of phospholipase C beta4 in thalamocortical relay nucleus leads to absence seizures. Proc Natl Acad Sci U S A. 2009;106:21912–21917.
27. Liu XB, MuÇoz A, Jones EG. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol.1998;395:450–465.
28. Muly EC, Maddox M, Smith Y. Distribution of mGluR1alpha and mGluR5 immunolabeling in primate prefrontal cortex. J Comp Neurol.2003;467:521–35.
29. Budziszewska B, Van Luijtelaar G, Coenen AM, et al. Effects of neurosteroids on spike-wave discharges in the genetic epileptic WAG/Rij rat. Epilepsy Res.1999;23–9.
30. Bouwman BM, Suffczynski P, Midzyanovskaya IS, et al. The effects of vigabatrin on spike and wave discharges in WAG/Rij rats. Epilepsy Res.2007;76:34–40.
31. Liu Z, Vergnes M, Depaulis A, et al. Evidence for a critical role of GABAergic transmission within the thalamus in the genesis and control of absence seizures in the rat. Brain Res. 1991;545:1–7.
32. Citraro R, Russo E, Gratteri S, et al. Effects of non-competitive AMPA receptor antagonists injected into some brain areas of WAG/Rij rats, an animal model of generalized absence epilepsy. Neuropharmacology 2006;51: 1058–1067.
33. Belelli D, Herd MB. The contraceptive agent Provera enhances GABA(A) receptor-mediated inhibitory neurotransmission in the rat hippocampus: evidence for endogenous neurosteroids? J Neurosci.2003;23:10013–10020.
34. Errington AC, Cope DW, Crunelli V. Augmentation of Tonic GABA(A) Inhibition in Absence Epilepsy: Therapeutic Value of Inverse Agonists at Extrasynaptic GABA(A) Receptors. Adv Pharmacol Sci.2011;2011:790–590.
35. Aronica E, Gorter JA, IjIst-Keizers H, et al. Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. Eur J Neurosci.2003;2106–18.
36. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. London: Academic (in press 2000)
37. van Luijtelaar ELJM, Coenen AML. Circadian rhythmicity in absence epilepsy in rats. Epilepsy Res.1988;2:331–336.
38. Smyk MK, Coenen A, Lewandowski MH, et al. Internal desynchronization facilitates seizures. Epilepsia 2012;53:1511–1518.
39. Ovchinnikov A, Lüttjohann A, Hramov A, et al. An algorithm for real-time detection of spike–wave discharges in rodents. J Neurosci Methods.2010;194:172–178.
40. van Rijn CM, Gaetani S, Santolini I, et al. WAG/Rij rats show a reduced expression of $CB_1$ receptors in thalamic nuclei and respond to the $CB_1$ receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike–wave discharges. Epilepsia 2010;51:1511–1521.




# 6 Is there a future for mGlu5 PAMs in absence epilepsy? A comparison with Ethosuximide

**Submitted as**

Gilles van Luijtelaar, Valerio D'Amore, Ines Santolini, Richard Ngomba

## Abstract

Ethosuximide is the drug of choice in the treatment of various types of absence seizures. However, there is room for other anti absence drugs considering that not all subjects (57%–74%) become seizure free and in about 47% of subjects ethosuximide therapy failed. New anti-absence drugs may target or modulate glutamatergic and or GABA-ergic neurotransmission, the key players in the circuitry involved in the cortico-thalamo-cortical oscillations responsible for the highly stereotyped spike-wave discharges (SWDs). The rat's somatosensory cortex contains a highly excitable focal region while tonic inhibition is dominant in the thalamus. Biochemical studies have shown that symptomatic WAG/Rij rats differ from age matched controls regarding increased mGlu5R expression in the somatosensory cortex and decreased mGlu1R and mGlu5R expression and function in the thalamus. Two group I positive allosteric modulators VU0360172 and RO0711401 dose-dependently suppressed SWDs in acute pharmacological studies, and both compound were effective in cortex and thalamus. Interestingly, the GABA reuptake blocker tiagabine reduced SWDs only in cortex, while the efficacy of ethosuximide is much higher in the highly excitable cortex than in the thalamus. It is therefore proposed that VU0360172 stimulates cortical GABA interneurons, which inhibit the highly excitable cortical neurons in the focal area and that in the thalamus, VU0360172 may reduce tonic inhibition. Group I PAMs might be further develop as an anti-absence drugs, while their preclinical profile in mesial temporal epilepsy models, another focal type of epilepsy, needs to be determined.


**Key words:** Group I mGluR; Positive allosteric modulators; absence epilepsy; genetic models; WAG/Rij rats; focal epilepsy; GABA; somatosensory cortex; antiepileptic drug development;

# Introduction

Childhood absence epilepsy (CAE) is the most common form of pediatric epilepsy, accounting for 10% to 17% of all cases. It typically begins between 4 and 8 years of age and is characterized by daily, frequent, brief staring spells, often with the eyes fluttering and the child being unresponsive to external stimuli. Children may experience dozens to hundreds of these episodes per day. Ethosuximide (ETX), dating back to the 1950s, and valproic acid (VPA) have been and still are the drug of choice in absence seizures for many decades, while a relative newly developed lamotrigine, was proposed and approved in the mid nineties [2]. It was only recently that the efficacy, safety and tolerability of the three compounds were compared in a large multi center study. A 2010 double-blind randomized, comparative controlled, including 446 patients, examined the efficacy and tolerability of ETX, valproic acid, and lamotrigine. At the week 16–20 visit, subjects on ETX (53%) and valproic acid (58%) had significantly higher freedom from failure rates than subjects on lamotrigine. Next, subjects on ETX had significantly less attention dysfunction compared to subjects on [3]. This combination of findings implied that ETX is the optimal initial monotherapy for CAE. Despite these rather favorable outcomes and ETX being the "winner" at week 16–20, ETX therapy failed in 47% of subjects: 14% due to seizures, 24% due to intolerable side effects, 13% withdrew from study. Epidemiologic cohort studies showed only freedom in the range from 21%–74%. In five prospective cohort studies, the proportion of seizure free subjects was 57%–74%. Although labeled a "benign" syndrome, the clinical course of CAE is variable and remission rates are far lower than in other classic benign idiopathic epilepsies such as Benign Rolandic Epilepsy [4]. Although a complete discussion of the adverse effects is beyond the scope of discussion, the reader is referred to a recent review of Gören and Onat [5], the usage of ETX has been associated with commonly observed dose-dependent side effects related to the gastrointestinal tract, central nervous system and hematopoietic adverse effects. Nausea, abdominal discomfort, vomiting, diarrhea, and anorexia are common at the onset of ETX therapy. Central nervous system effects include drowsiness, dizziness, hiccups, fatigue, insomnia, tiredness, headache, and psychotic behaviors. ETX has been also associated with a wide variety of idiosyncratic reactions, including allergic dermatitis, rash, Stevens-Johnson syndrome, systemic lupus erythematosus, lupus-like syndrome, serum sickness reaction, agranulocytosis, and aplastic anemia [3,6]. The hematopoietic effects include leukopenia, eosinophilia, and pancytopenia [7]. A new dimension to the treatment of absence epilepsy has been added by the proposal that ETX, first in WAG/Rij rats, subsequently also in GAERS, has besides antiabsence effects [8] also antiepileptogenic effects when treatment started early and lasted 4 months [9,10,11,12] and that the comorbidity of absence epilepsy with a mild form of depression-like behavior, typically for the WAG/Rij strain [13] did not occur as a consequence of anti epileptogenesis by ETX [14]. Most relevant, from a theoretical but also from a translational point of view is that remission is more than likely in high

compliance absence patients that were treated with ETX [15]. It can be concluded that ETX is the most precious tool in the treatment of absence epilepsy, that it is certainly not without problems, including the relative high failure rate, and its putative adverse effects suggests that there must be room for a new and better anti-absence drugs. Moreover, ETX is the drug of choice, new and better antiabsence drugs must be compared with ETX.

## The assumed working mechanisms of ETX

It has long been thought that the action of ETX is blocking It currents in thalamic-cortical (TC) relay cells and, consequently, the burst firing mode of TC relay cells. The burst firing of TC cells was assumed to be the neurophysiologic substrate of SWD activity in cortico-thalamo-cortical pathways. These ideas were developed in the beginning of the nineties of the previous century based on *in vitro* neurophysiological studies using voltage-clamp techniques in acutely isolated neurons of the ventrobasal complex from rats and guinea pigs. Therapeutically relevant concentrations ETX induced a reduction of the low threshold $Ca^{2+}$ current which was most pronounced at more-hyperpolarized potentials first in the relay nuclei with no change in its kinetics or steady-state properties [16,17,18], later this was also found for the reticular thalamic nucleus (RTN) [19]. It was generally felt that virtually all thalamic neurons are endowed with a prominent low-threshold $Ca^{2+}$ conductance that is of sufficient magnitude to generate a low-threshold $Ca^{2+}$ spike and the accompanying burst firing mode of thalamo-cortical cells [20,21,22]. This conductance is crucial in the generation of normal thalamo-cortical oscillations such as sleep spindles [23], and in the occurrence of delta waves, typical for deep slow sleep [24,25]. The possibility was raised that it may also play a key role in the development of pathological SWDs and this idea was thought to be a sufficient explanation for the working mechanism of ETX in reducing SWDs in absence epilepsy patients. However, ETX also decreases persistent $Na^+$ and $Ca^{2+}$-activated $K^+$ currents in thalamic and layer V cortical pyramidal neurons [26,27] which may explain also the decrease in burst and the increase in tonic firing. These results cast doubts on the hypothesis that a reduction of IT in thalamic neurons underlies the therapeutic action of this anti-absence medicine [26]. In addition, there is evidence that in a genetic absence epilepsy rat model ETX reduces cortical γ-aminobutyric acid (GABA) levels. Also, elevated glutamate levels in the primary motor cortex of rats with absence epilepsy (but not in normal animals) are reduced by ETX. In addition, whole-cell patch-clamp studies revealed an increase in spontaneous GABA release by ETX concurrent with no change in glutamate release at synapses in the rat entorhinal cortex *in vitro* [28]. This was reflected in a substantial rise in the ratio of network inhibition to excitation, and a concurrent decrease in excitability of neurons embedded in this network. These authors concluded that ETX directly elevates synaptic inhibition in the cortex next to its well established effects on ion channels, and that both factors contribute to its well known anti-absence effects. Interestingly, an increase in synaptic inhibition of cortical cells was also found after tiagabine by the same authors.

This notion is relevant considering that there is now good evidence that generalized absence seizures in the genetic models, but also in a by systemic administration of a low dose of PTZ induced absence-like seizures, are initiated at a cortical focus in the deep layers of the somatosensory cortex [29,30]. Subsequent studies revealed that ETX exerted an immediate and strong anti-absence action when injected in the somatosensory cortex and not the cortex in general [31,32]. Moreover, *In vivo* studies showed that the majority (60%) of cat TC neurons are completely silent during SWDs [33] and in GAERS, a well characterized and validated rat genetic model of absence epilepsy, no low threshold Ca2+ spikes (LTSs) are recorded in TC neurons during the majority (90%) of SWDs [34,35]. However, hyperpolarized mediated LTSs and the burst firing mode were seen in RTN during SWDs in GAERS [35]. Microinfusion of ETX into VB produced only a weak and delayed anti-absence effect while the effects in the RTN were of a larger magnitude. In all, the outcomes of also these studies questioned the notion that ETX exerts its therapeutic effect for a large extent at the thalamic level [36].

Monotherapy often fails and seizure control can be only achieved with the administration of polytherapy. Valproic acid and ETX were singly and also co administered in rats of the WAG/Rij strain. The incidence of SWDs in the electroencephalogram (EEG) was the read-out variable. The median effective doses (ED50 values) of the two drugs in this model were 121 and 21.5 mg/kg for valproic acid and ETX, respectively [37]. When both agents were administered together, the interaction between the two agents was shown to be infraadditive: the combination was less effective in diminishing the incidence of SWDs in WAG/Rij rats than could be anticipated and based on the net effect of the drugs administered alone. This is indirect evidence that the two anti absence drugs share some common cellular or molecular mechanism, perhaps blocking $Na^+$ channels.

## New anti-absence drugs

The search for new anti-absence drugs has been concentrated on drugs that target the circuitry in which the for absence epilepsy characteristic SWDs are elicited, maintained and aborted. For typical absence epilepsy is undoubtedly the reciprocally interconnected cortico-thalamo-cortical network including the RTN [38,39,40,41], for atypical absence epilepsy it is more likely a hippocampal, thalamo-cortical circuit [42]. GABA and glutamate are the major neurotransmitter systems within the interconnected cortex and thalamus. Therefore, it seems logic to investigate the role of these neurotransmitter systems on the incidence of SWDs. Our group has done most of the experiments in a well characterized and validated 5 model of absence epilepsy, rats of the WAG/Rij strain [43]. The model has construct, face, and pharmacological validity [39,44,45]. In the mid nineties, drugs affecting ionotropic glutamate receptors were in the focus of interest and all subclasses of antagonists reduced SWD while agonists enhanced SWD in our genetic absence model [45,46,47,48]. However, many of the ionotropic receptor antagonists had toxic effects and their role in seizure control has not led to the successful development of new antiabsence or anticonvulsant drugs.

Since targeting mGluRs might be less toxic than targeting ionotropic glutamate receptors, we decided to focus on the role of mGluRs, also considering that drugs targeting mGluR have been shown to exert pro or antiepileptic effects in various seizure and epilepsy models [49]. More specifically: orthosteric antagonists of Group I mGluR reduce motor seizures induced by sound stimulation in DBA mice as well as non-convulsive (absence) seizures in the lethargic mice model [50,51], the latter is a genetic absence model with ataxia and hypoactivity, while the non-selective orthosteric agonist DHPG targeting both type 1 and 5 receptors, enhances convulsions [52,53]. The role of orthosteric agonists on non-convulsive epilepsy has not been investigated. Interestingly, besides orthosteric agonists and antagonists also more selective allosteric modulators (PAMs and NAMs) are currently developed and available, which might be even more subtle in inducing changes. A second reason was that the mGluRs are widely distributed in the cortico-thalamo-cortical networks [54], the brain circuitry in which SWD originate, are maintained, and end [40]. This network consists of a large number of subtypes of mGluR, and they are selectively present in different parts of the circuitry, offering possibilities for selective targeting disease modified (sub)systems. Here we focus on specifically the role of Group I mGluRs receptor with its two subtypes (I and 5) in the pathogenesis of absence epilepsy and whether drugs affecting this system might be putative anti-absence drugs. It is known that Group I mGluRs are involved in absence epilepsy because thalamo-cortical -limited knockdown as well as whole-animal knockout of PLCβ4, a protein involved in the Group I signaling pathway, induced spontaneous SWDs in mice with simultaneous behavioral arrests. In addition, the susceptibility to drug-induced SWDs was increased in these knock-out mice, indicating that the deletion of thalamic PLCβ4 leads to the genesis of absence seizures [55]. Interestingly, PLCβ4 co localizes with mGlu1 receptors, and mediates mGlu1 receptor signaling in thalamic nuclei [56,57]. Moreover, also the studies by Chapman et al. [50,51] suggest a role of these receptors in non-convulsive seizures.

## Neurochemical studies

As mentioned above, mGluRs are widely distributed in the cortico-thalamo-cortical network [54]. In the thalamus, group I mGluRs are located on relay neurons in the ventrobasal thalamus postsynaptic to the cortical inputs [58,59,60]. Moderate-to-low mGlu5 receptor mRNA and protein levels are expressed in RTN neurons [59,61], whereas these neurons do not express mGlu1 receptor mRNA [62]. mGlu1 receptors in the cortex are located 6 postsynaptically on GABAergic interneurons [63], and mGlu5 receptors are expressed by pyramidal neurons postsynaptic to thalamo-cortical projections [64], as well as by interneurons [59,65]. Group I mGluRs are coupled to Gq/G11 proteins and their activation stimulates polyphosphoinositide hydrolysis with formation of inositol-1,4,5-trisphosphate and diacylglycerol, and also regulate the activity of different types of $Ca^{2+}$ and $K^{+}$ channels [66].

Several studies of both expression and function were performed in the WAG/Rij in order to assess the biochemical role of mGluRs receptors in the pathophysiology of non-convulsive epilepsy. Relevant is that in the WAG/Rij rat absence model the SWDs start to become present in the cortical EEG from 2-3 months of age onwards. The analysis of expression of mGluRs was carried out by using symptomatic WAG/Rij rats (minimally 6 months of age) and pre-symptomatic WAG/Rij rats (not more than 2 months of age) which were compared with age-matched control non epileptic ACI rats. Various part of the cortico-thalamo-cortical network, such as RTN, ventrobasal thalamic nuclei, somatosensory cortex and motor cortex were inspected [67,68]. The functional activity of Group I mGluRs receptors was examined by using an *in vivo* method that allows measurements of agonist-stimulated PI hydrolysis after incorporation of [3H]inositol into the phospholipids of living rats [69]. mGlu1 or mGlu5 receptor function was evaluated in the thalamus and or in the somatosensory cortex of symptomatic WAG/Rij rats as compared to age matched controls.

Both the expression and function of mGlu1 and mGlu5 receptors were reduced in the thalamus of symptomatic WAG/Rij rats compared to age-matched non-epileptic ACI rats. Lower levels of Group I subtype protein receptors in the thalamus of the symptomatic WAG/Rij rats were confirmed by immunohistochemical analysis [67]. Moreover, these data showed that the reduced expression of mGlu1 receptors found in symptomatic WAG/Rij rats was observed in the ventrobasal complex, that correspond to dorsal and medial nucleus, but not in the ventroposterolateral thalamus. Furthermore, no mGlu1 receptor mRNA was observed in the RTN of the rats. No change in mGlu1 receptor signaling was detected in the somatosensory cortex of symptomatic WAG/Rij rats compared to age-matched control ACI rats. In contrast, the expression of mGlu5 receptors was increased either in the somatosensory and motor cortex of symptomatic WAG/Rij rats, as assessed by immunohistochemical and western blot analysis [68].

In all, these neurochemical data demonstrate that absence epilepsy in this genetic model is accompanied by significant changes in expression and function of group I mGluR in for absence epilepsy relevant parts of the SWD generating circuit.



## Acute and subchronic Pharmacological studies

Considering our aim to evaluate putative new treatments for absence epilepsy, first the effects of a selective mGluR group I subtype 1 PAM on the occurrence of spontaneous absence seizures were investigated. Adult symptomatic rats of the WAG/Rij strain were treated systemically with the compound RO0711401 (3, 10 or 30 mg/kg, s.c.), a selective PAM at mGlu1 receptors [70]. The outcomes indicated that the dose of 3 mg/kg of RO0711401 could only abolish the early stress-related increase in the incidence of SWDs; a dose of 10 mg/kg was required for a substantial reduction in the incidence of SWDs; and a dose of 30 mg/kg could reduce the incidence as well as the mean duration of trains of SWDs. To further demonstrate a protective role for mGlu1 receptors against SWDs, the

same rats were systemically injected with compound JNJ16259685 (2.5 or 5 mg/kg, i.p.), which behaves as a selective NAM of mGlu1 receptors. Treatment with JNJ16259685 increased the incidence of SWDs in a dose-dependent manner [67].

Group I (mGluR1 and mGluR5) receptors show a different pattern of distribution in the C-T-C network, which suggests a distinct rather than complementary functions of these two receptor subtypes. Therefore our pharmacological investigation was extended to mGlu5 receptors. The pharmacological enhancement of mGluR5 activity with the PAM VU0360172 at doses 3 and 10 mg/kg, s.c., known to be centrally active [71], caused a robust and dose-dependent reduction in the incidence and mean duration of SWDs. The acute treatment with MTEP (10 or 30 mg/kg, i.p.), a selective NAM of mGlu5 receptors [72,73], did not change the incidence and mean duration [68].

From a therapeutic standpoint it is important to establish whether tolerance developed to the action of these putative anti absence drugs. Effective doses of VU0360172 (3 mg/kg, s.c.) and RO0711401 (10 mg/kg, s.c.) were administered in rats twice daily for ten days and the incidence of SWD was daily determined. As expected [67,68] both mGlu1 and mGlu5 receptor PAMs suppressed the incidence of SWDs in the first 2 days of the treatment. While the anti-absence effect of the mGlu5 receptor PAM (VU0360172), persisted largely over the 10 day administration period with only a small sign of tolerance. In contrast, rats quickly developed complete tolerance to RO0711401 since the 3rd day of treatment. The results are presented in Figure 1.

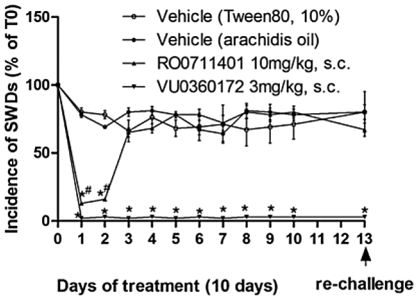



**Figure 1** Tolerance towards twice daily s.c. administration of VU360172 (3 mg/kg) and RO711401 (10 mg/kg) on incidence of spike-wave discharges (SWDs) in adult (> 6 month) WAG/Rij rats during baseline (hour 0) and during first hour post injection at Day 1-10 and Day 13 (rechallenge), control rats were given vehicle. Rats injected with VU0360172 showed an antiabsence effect throughout all injection days without clear signs of tolerance; in contrast, RO711401 was effective on Day 1 and 2 only and it lost completely its antiabsence action at Day 3. This lack of effects persisted throughout the remainder of the study, including during the rechallenge after 2 days of withdrawal. Depicted are means ± S.E.M. of incidence of SWDs, 8-9 animals per group (Adapted After [74]).

Next it was wondered whether chronic treatment with RO0711401 or VU0360172 would cause desensitization of mGlu1 and mGlu5 receptors both in symptomatic WAG/Rij rats and in non-epileptic age matched Wistar control rats [74]. Chronic administrations of VU0360172 increased the expression of mGlu5 receptors in the thalamus and in the cortex in WAG/Rij rats without changing the expression of mGlu1a receptors. Treatment with RO0711401 enhanced the expression of both mGlu1a and mGlu5 receptors in thalamus and cortex in WAG/Rij rats. Opposite data were obtained in non-epileptic Wistar rats, in which repeated injections of the two PAMs down-regulated the expression of mGlu1a and mGlu5 receptors. RO0711401 changed the expression of both the mGlu1a and the mGlu5 receptors in WAG/Rij and Wistar rats, whereas VU0360172 selectively changed the expression of mGlu5 receptors only [74].

All these outcomes contribute to highlight the key role of the Group I mGluRs in the pathophysiology of absences seizures, at least in the spontaneous absence seizures model WAG/Rij rats. Moreover, they suggest that chronic administration of group I PAMs may change the expression of mGlu1a and mGlu5 in cortex and thalamus, that these changes might be different for the two different PAMs and that the genetic context may also be crucial for the changes in receptor function after repeated administration.

## Local injection studies

The next step was a C-T-C circuit demarcation strategy adopted by independent bilateral intra-cortex or intra-thalamus (ventral basal complex) micro-infusions to investigate site-specific effects on the regulation of SWDs WAG/Rij rats received micro-infusion with RO0711401 or VU030172. The two drugs were equally effective in reducing the incidence of SWDs when injected into the cortex. Both drugs were also effective in reducing SWDs incidence when they were infused in the thalamus, although the mGlu5 PAM VU036012 displayed a somewhat larger efficacy than the mGlu1 PAM RO0711401, when injected into the thalamus [75]. It is well known that not only a dysfunction in glutamatergic neurotransmission is known to be one of the causes responsible for the initiation and spread of seizures, but also GABAergic neurotransmission. Moreover, glutamate and GABA interact at many locations within the T-C-T network. Therefore first the effects of tiagabine alone, a GABA reuptake inhibitor at the neuronal or glial GAT-1 transporter, micro-injected in cortex and thalamus were investigated. Cortical administration of tiagabine resulted in a dose-dependent decrease in the incidence of SWDs, an effect similar to the effects of the PAMs of mGluR. In contrast: intra-thalamic injections showed a dose-dependent increase in the incidence of SWDs, an effect opposite to that obtained with the PAMs.

Next, the interaction between GABA and glutamate was investigated. More precisely, it was established whether and in which direction an increased availability of (extra) synaptic GABA influences responses to VU0360172 in the thalamus and cortex. Combined micro injections of VU0360172 and tiagabine were carried out. Co-administration of VU0360172 and tiagabine reduced the incidence in the SWDs when injected in the cortex

and some indications were obtained for subadditive effects. In contrast, complete opposite effects were observed after the same co-administration in the thalamus: first a decrease, which was followed by an increase in the incidence of SWD, suggesting that the effects of VU0360172 were completely blocked in the presence of increased GABA [75]. In all the above mentioned experiments the behavior of the rats was quantified with the aid of a calibrated infrared movement detector; it allowed the quantification of the amount of bodily activity during the EEG recording session. In none of the experiments described above, VU0360172 affected the behavior of the rats.

It can be concluded that these pharmacological studies demonstrate dose dependent SWD decreasing effects of both PAMs, minimal tolerance in a twice daily 10 day administration protocol of VU0360172 and the quick development of tolerance to RO0711401. Local injections of both PAMs demonstrated that both compounds are effective in cortex and thalamus. Effects on behavior were not noticed. In all, VU0360172 has a good preclinical profile in the WAG/Rij absence model.

# Discussion

The preclinical profile of VU0360172 as an antiabsence drug, as described here, seems promising, although only a few studies have investigated its effects. ETX has a much longer tradition, it has the best clinical profile of all anti absence drugs, although recent reviews also emphasize the need for other and newer treatment options. Many earlier *in vivo* and *in vitro* neurophysiologic studies in mostly healthy individuals of different species such as rats, cats, and ferrets, e.g. [19,20,76,77] have found that ETX acts in the thalamus by blocking Ca2+ channels, both in the relay nuclei and in the RTN. The comparative cortex-thalamus local injection studies with ETX in the GAERS absence model have yielded a somewhat different pattern since they have shown that ETX is more effective in the somatosensory cortex than in the thalamus. Moreover, this cortical effect of ETX was restricted to the focal region in the somatosensory cortex, while in the thalamus ETX showed a less large effect in both the VB nuclei and in the RTN [31,36]. A compilation of the WAG/Rij and GAERS data regarding the local administration of ETX and VU0360172 in cortex and thalamus is presented in Figure 2.

Next to blocking pyramidal cortical cells within layer V–VI of the facial somatosensory cortex in GAERS [32], most likely via a specific attenuation of the noninactivating $Na^+$ and $Ca^{2+}$-activated $K^+$ currents [26,27], ETX might also facilitate GABA-ergic neurotransmission [28]. Interestingly, VU0360172 and RO0711401 were two drugs that were equally effective in suppressing SWDs in the somatosensory cortex and ventral basal part of the thalamus, and this was now found in the WAG/Rij model. This suggests that VU0360172 and RO0711401 may target both locations equally effective and therefore we propose that this factor may contribute to successful seizure suppression of both of both mGluR Group I

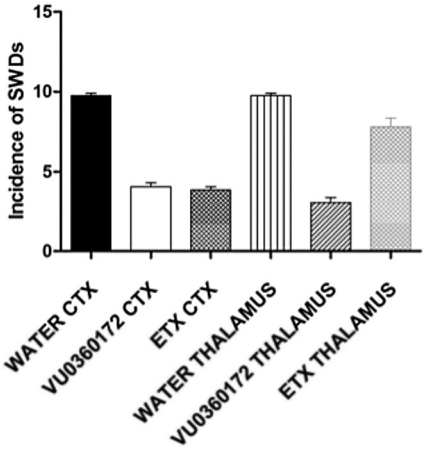



**Figure 2** Local cortical and thalamic injections of ethosuximide and VU360172 on incidence of spike-wave discharges (SWDs) in 30 min after local administration in facial area of somatosensory cortex and the ventral basal complex of the thalamus (data from [31,75]).

PAMs. At first glance the SWD reducing effects of VU0360172 and RO0711401 seems puzzling considering that group I agonists and PAMS enhance glutamatergic neurotransmission [78] and that at least the glutamatergic (ionotropic) agonists have proabsence effects [46,47,48], as was established in the WAG/Rij model. Our local injections of the PAMs in cortex and thalamus and the comparative study with tiagabine also in WAG/Rij rats showed two interesting facts. The first was that both PAMs suppressed SWD when injected in the thalamus. It was thought that the SWD inhibiting effects of the PAMS in the thalamus were due to an increased glutamatergic excitation which inhibited the tonic inhibition, one of the assumed causes for the occurrence of SWD [79]. Second, cortical injections with the PAMs also suppressed SWDs, but here the cause of epilepsy is an increased local excitation, a second reason for SWD occurrence [30,39,80]. Both tiagabine and VU0360172 showed the same SWD reducing effect after local cortical microinjections, which were ascribed to the stimulating effects of VU0360172 on cortical GABA-interneurons, which released GABA when stimulated by VU0360172, mimicking the net effects of the cortical microinfusion of the GABA reuptake blocker tiagabine itself [75].

It needs to be added that the results, as obtained regarding the direction of the effects of the Group I PAMs and NAMs as described here, were opposite to what has been described in the lethargic mice model [50,51]. It is obvious that the effects could be dependent on the genotype (WAG/Rij rats versus lethargic mice). More specifically, our biochemical studies have clearly revealed that absence epilepsy in WAG/Rij rats is

accompanied by, among others [39,48,81], significant changes in expression and function of group I mGluR in cortex and thalamus [67,68],so in the for absence epilepsy most relevant parts of the SWD generating circuit and it is by no means clear that the same SWD reducing effects of group I PAMs will be obtained in other genetic absence models. On the other hand, the WAG/Rij model has predictive validity and until now there are no false positive or false negatives [45]. The lethargic mice has a rather complex phenotype (absence seizures, chronic ataxia, hypoactivity and transients attacks of severe dyskinetic motor behaviour) and also in this model in situ hybridization and Western blot analyses have indicated large increase in GAD(67) expression (mRNA and protein) in thalamic cells and disturbances of the GABAB receptor system with an involvement of T-type voltage gated $Ca^{2+}$ channels [82].

From a drug developmental perspective it is important that in none of our studies with VU0360172 and RO0711401 qualitative and quantitative changes in motor behaviour of the animals were found, although in each of them the spontaneous behaviour was quantified with an infrared movement detector. Moreover, the lack of behavioural changes suggest also that the anti-absence effects of the PAMs are not secondary to changes in wake behavior, or can be explained by sleep related variables, as far as the latter could be inferred from our quantitative behavioral measurements. Interestingly, VU0360172 did not affect spontaneous motor activity in an open field but it did attenuate dose-dependently hyperlocomotion induced by the psychotomimetic amphetamine [83]. Tolerance towards the antiabsence action to a ten day twice daily drug regime was minimal with VU0360172, in contrast to RO0711401. With the latter drug tolerance was absent on the first 2 days (in fact the acute antiabsence action lasted up to 6 hours) and was complete after 3 days.

Neurotoxic studies with higher doses of VU0360172, such as the measurement of stereotyped motor coordination tasks, such as balancing on a rotarod, but also towards sedative and analgesic effects obviously need to be done. More challenging is to establish effects on cognition. Fortunate is that Group I PAM's were already proposed for the treatment of psychosis and cognitive disturbances in schizophrenia patients, also since Group I PAMs enhance certain forms of synaptic plasticity via LTD and LPT and learning and memory processes [84]. These latter properties were ascribed to the ability of PAMs to indirectly potentiate the function of NMDA receptors; our cortical injection studies suggest that the action group I PAMs might in addition to this well known effect, also facilitate cortical GABA-ergic neurotransmission.

Antiepileptic drugs are often given in combination considering than monotherapy might often be insufficient for obtaining full seizure control. However, many antiepileptic drugs are enzyme inducing and substantially lower the efficacy of other medications. ETX showed infraadditive effects with valproic acid in WAG/Rij rats. Combination studies of PAMs with ETX and valproic acid are indicated. Considering that antiepileptogenesis can be achieved with ETX and levitiracetam in the genetic absence models, it is also indicated

that it important to investigate whether antiepileptogenesis is also possible with Group I PAMs.

The local cortical injection studies with Group I PAMS strongly suggest that these PAMs deserve to be investigated in other types of epilepsy models, in which seizures have their origin in a brain region in which glutamate and GABA interact, such as in various types of mesial Temporal Lobe epilepsy models and in which local increases glutamatergic activity cannot be sufficiently inhibited. Also in these models VU0360172 may enhance the diminished GABA mediated inhibitory neurotransmission.

VU0360172 does not fully interact in fully competitive manner wi with a prototypic allosteric binding site, in contrast to some other recently synthetized mGlu5 PAMs (Rook et al., 2015); however whether this is crucial or not for its actions *in vivo*, in our case its anti-absence action, remains to be elucidated.

# References


[1] Wallace SJ. Use of ethosuximide and valproate in the treatment of epilepsy. Neurol Clin. 1986; 4: 601-616.

[2] Frank LM, Enlow T, Holmes GL, Manasco P, Concannon S, Chen C, Womble G, Casale EJ. Lamictal (lamotrigine) monotherapy for typical absence seizures in children. Epilepsia. 1999; 40: 973-979.

[3] Glauser TA, Cnaan A, Shinnar S, Hirtz DG, Dlugos D, Masur D et al. , Ethosuximide, valproic acid, and lamotrigine in childhood absence epilepsy. N Engl J Med 2010; 362: 790–799.

[4] Teoh PC, Chan HL. Lupus-scleroderma syndrome induced by ethosuximide. Arch Dis Child 1975; 50: 58–61.

[5] Gören MZ, Onat F. Ethosuximide: from bench to bedside. CNS Drug Rev. 2007; 13: 224-39.

[6] Tenney JR, Glauser TA. The current state of absence epilepsy: can we have your attention? Epilepsy Curr. 2013; 13 : 135-140.

[7] Posner EB, Mohamed K, Marson AG. A systematic review of treatment of typical absence seizures in children and adolescents with ethosuximide, sodium valproate or lamotrigine. Seizure 2005; 14: 117–122.

[8] van Luijtelaar G, Wiaderna D, Elants C, Scheenen W. Opposite effects of T- and L-type Ca2+ channels blockers in generalized absence epilepsy. Eur J Pharmacol. 2000; 20: 406: 381-389.

[9] Blumenfeld H, Klein JP, Schridde U, Vestal M, Rice T, Khera DS et al. Early treatment suppresses the development of spike-wave epilepsy in a rat model. Epilepsia 2008; 49: 400-409.

[10] van Luijtelaar G, Mishra AM, Edelbroek P, Coman D, Frankenmolen N, Schaapsmeerders P et al. Anti-epileptogenesis: Electrophysiology, diffusion tensor imaging and behavior in a genetic absence model. Neurobiol Dis. 2013; 60: 126-138.

[11] Russo E, Citraro R, Scicchitano F, De Fazio S, Di Paola ED, Constanti A et al. Comparison of the antiepileptogenic effects of an early long-term treatment with ethosuximide or levetiracetam in a genetic animal model of absence epilepsy. Epilepsia. 2010; 51: 1560-1569.

[12] Dezsi G, Ozturk E, Stanic D, Powell KL, Blumenfeld H, O'Brien TJ et al. Ethosuximide reduces epileptogenesis and behavioral comorbidity in the GAERS model of genetic generalized epilepsy. Epilepsia. 2013; 54: 635-643.

[13] Sarkisova K, van Luijtelaar G. The WAG/Rij strain: a genetic animal model of absence epilepsy with comorbidity of depression [corrected]. Prog Neuropsychopharmacol Biol Psychiatry. 2011; 35: 854-876. Review. Erratum in: Prog Neuropsychopharmacol Biol Psychiatry. 2012; 36: 212.

[14] Sarkisova KY, Kuznetsova GD, Kulikov MA, van Luijtelaar G. Spike-wave discharges are necessary for the expression of behavioral depression-like symptoms. Epilepsia. 2010; 51: 146-160.

[15] Berg AT, Levy SR, Testa FM, Blumenfeld H. Long-term seizure remission in childhood absence epilepsy: might initial treatment matter? Epilepsia 2014; 55: 551-557.

[16] Coulter DA, Huguenard JR, Prince DA. Characterization of ethosuximide reduction of low-threshold calcium current in thalamic neurons. Ann Neurol. 1989; 25: 582-593.

[17] Macdonald RL, Kelly KM. Antiepileptic drug mechanisms of action. Epilepsia. 1993; 34 Suppl 5: S1-8.

[18] White HS. Comparative anticonvulsant and mechanistic profile of the established and newer antiepileptic drugs. Epilepsia. 1999; 40 S5: S2-10.

[19] Huguenard JR, Prince DA. Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. J Neurosci 1994; 14: 5485–5502.

[20] Deschênes M, Paradis M, Roy JP, Steriade M. Electrophysiology of neurons of lateral thalamic nuclei in cat: resting properties and burst discharges. J Neurophysiol. 1984; 51: 1196-1219.

[21] Jahnsen H, Llinás R. Electrophysiological properties of guinea-pig thalamic neurones: an in vitro study. J Physiol. 1984; 349: 205-26.

[22] Jahnsen H, Llinás R. Voltage-dependent burst-to-tonic switching of thalamic cell activity: an in vitro study. Arch Ital Biol. 1984; 122: 73-82.

[23] Steriade M, Llinás RR.The functional states of the thalamus and the associated neuronal interplay. Physiol Rev. 1988; 68: 649-742.

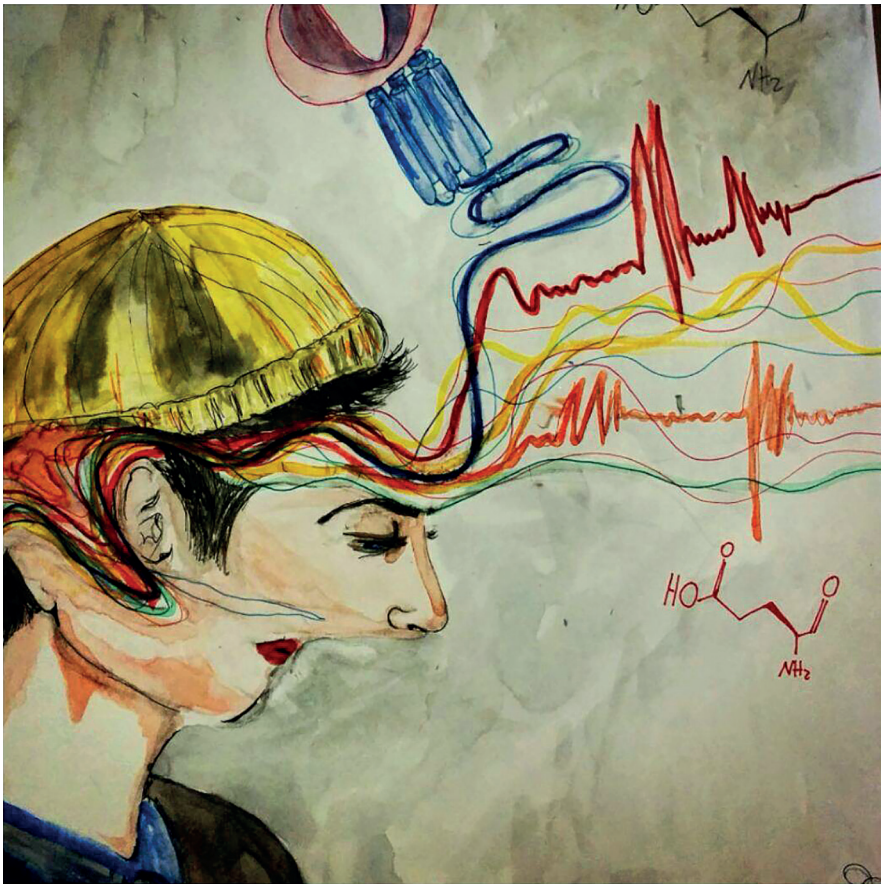
[24] Domich L, Oakson G, Steriade M. Thalamic burst patterns in the naturally sleeping cat: a comparison between cortically projecting and reticularis neurones. J Physiol Lond. 1986; 379: 429- 449.

[25] Crunelli V, David F, Leresche N, Lambert RC. Role for T-type Ca2+ channels in sleep waves. Pflugers Arch. 2014; 466: 735-745.

[26] Leresche N, Parri HR, Erdemli G, Guyon A, Turner JP, Williams, SR et al. On the action of the anti-absence drug ethosuximide in the rat and cat thalamus. J Neurosci. 1998; 18: 4842–4853.
[27] Crunelli V, Leresche N. Childhood absence epilepsy: genes, channels, neurons and networks. Nat Rev Neurosci. 2002; 3: 371-382.
[28] Greenhill SD, Morgan NH, Massey PV, Woodhall GL, Jones RSG. Ethosuximide modifies network excitability in the rat entorhinal cortex via an increase in GABA release. Neuropharmacol. 2012: 62; 807-814.
[29] Meeren HK, Pijn JP, van Luijtelaar EL, Coenen AM, Lopes da Silva FH. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci. 2002; 22: 1480-1495.
[30] Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S. Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. J Neurosci. 2007; 27: 6590-6599.
[31] Manning JP, Richards DA, Leresche N, Crunelli V, Bowery NG. Cortical-area specific block of genetically determined absence seizures by ethosuximide. Neuroscience. 2004; 123: 5-9.
[32] olack PO, Charpier S. Ethosuximide converts ictogenic neurons initiating absence seizures into normal neurons in a genetic model. Epilepsia. 2009; 50: 1816-1820.
[33] Steriade M, Contreras D. Relations between cortical and thalamic cellular events during transition from sleep patterns to paroxysmal activity. J Neurosci. 1995; 15: 623-642.
[34] Pinault D, Leresche N, Charpier S, Deniau JM, Marescaux C, Vergnes M, Crunelli V. Intracellular recordings in thalamic neurones during spontaneous spike and wave discharges in rats with absence epilepsy. J Physiol. 1998; 509: 449-456.
[35] Slaght SJ, Leresche N, Deniau JM, Crunelli V, Charpier S. Activity of thalamic reticular neurons during spontaneous genetically determined spike and wave discharges. J Neurosci. 2002; 22: 2323-2334.
[36] Richards DA, Manning JP, Barnes D, Rombola L, Bowery NG, Caccia S et al. Targeting thalamic nuclei is not sufficient for the full anti-absence action of ethosuximide in a rat model of absence epilepsy. Epilepsy Res. 2003; 54: 97-107.
[37] van Rijn CM, Sun MS, Deckers CL, Edelbroek PM, Keyser A, Renier W, Meinardi H. Effects of the combination of valproate and ethosuximide on spike wave discharges in WAG/Rij rats. Epilepsy Res. 2004; 59: 181-189.
[38] Blumenfeld H. Cellular and network mechanisms of spike-wave seizures. Epilepsia. 2005; 46 Suppl 9: 21-33.
[39] van Luijtelaar G, Sitnikova E. Global and focal aspects of absence epilepsy: the contribution of genetic models. Neurosci Biobehav Rev. 2006; 30: 983-1003.
[40] Lüttjohann A, van Luijtelaar G. Dynamics of networks during absence seizure's on- and offset in rodents and man. Front Physiol. 2015; 6: 16.
[41] Stefan H, Lopes da Silva FH. Epileptic neuronal networks: methods of identification and clinical relevance. Front Neurol. 2013; 4: 8.
[42] Onat FY, van Luijtelaar G, Nehlig A, Snead OC 3rd. The involvement of limbic structures in typical and atypical absence epilepsy. Epilepsy Res. 2013; 103: 111-123.
[43] van Luijtelaar EL, Coenen AM. Two types of electrocortical paroxysms in an inbred strain of rats. Neurosci Lett. 1986; 70: 393-397.
[44] Coenen AM, Van Luijtelaar EL Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav Genet. 2003; 33: 635-655.
[45] Depaulis A, van Luijtelaar G. Genetic models of absence epilepsy in the rat. In: Pitkänen A, Schwartkroin PA, Moshé SL (Eds) Models of Seizures and Epilepsy. Elsevier, Amsterdam, pp 233–248, 2006.
[46] Peeters BW, Ramakers GM, Ellenbroek BA, Vossen JM, Coenen AM. Interactions between NMDA and nonNMDA receptors in nonconvulsive epilepsy in the WAG/Rij inbred strain. Brain Res Bull. 1994; 33: 715-718.
[47] Jakus R, Graf M, Ando RD, Balogh B, Gacsalyi I, Levay G et al. Effect of two noncompetitive AMPA receptor antagonists GYKI 52466 and GYKI 53405 on vigilance, behavior and spike-wave discharges in a genetic rat model of absence epilepsy. Brain Res. 2004; 1008: 236-244.
[48] van Luijtelaar G., Zobeiri M. Progress and Outlooks in a Genetic Absence Epilepsy Model (WAG/Rij). Curr Med Chem. 2014; 21: 704-721.
[49] Alexander GM, Godwin DW. Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res. 2006; 71: 1-22.
[50] Chapman AG, Yip PK, Yap JS, Quinn LP, Tang E, Harris JR et al. Anticonvulsant actions of LY 367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid). Eur J Pharmacol. 1999; 368: 17-24. 16

[51] Chapman AG, Nanan K, Williams M, Meldrum BS. Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacol. 2000; 39: 1567-1574.
[52] Conn PJ, Pin JP. Pharmacology and functions of metabotropic glutamate receptors. Ann Rev Pharmacol Toxicol. 1997; 37: 205-237.
[53] Thomsen C, Dalby NO. Roles of metabotropic glutamate receptor subtypes in modulation of pentylenetetrazole-induced seizure activity in mice. Neuropharmacol. 1998; 37: 1465–1473.
[54] Ngomba RT, Santolini I, Salt TE, Ferraguti F, Battaglia G, Nicoletti F et al. Metabotropic glutamate receptors in the thalamocortical network: strategic targets for the treatment of absence epilepsy. Epilepsia. 2011; 52: 1211-1222.
[55] Cheong E, Zheng Y, Lee K, Lee J, Kim S, Sanati M et al. Deletion of phospholipase C beta4 in thalamocortical relay nucleus leads to absence seizures. Proc. Natl. Acad. Sci. USA 2009; 106: 21912-21917.
[56] Miyata M, Kashiwadani H, Fukaya M, Hayashi T, Wu D, Suzuki T et al. Role of thalamic phospholipase C[beta]4 mediated by metabotropic glutamate receptor type 1 in inflammatory pain. J Neurosci. 2003; 23: 8098-8108.
[57] Watanabe M, Nakamura M, Sato K, Kano M, Simon MI, Inoue Y. Patterns of expression for the mRNA corresponding to the four isoforms of phospholipase Cbeta in mouse brain. Eur J Neurosci. 1998; 10: 2016-2025.
[58] Ferraguti F, Crepaldi L, Nicoletti F. Metabotropic glutamate 1 receptor: current concepts and perspectives. Pharmacol Rev. 2008; 60: 536-581.
[59] Romano C, Sesma MA, McDonald CT, O'Malley K, Van den Pol AN, Olney JW. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J Comp Neurol. 1995; 355: 455-469.
[60] Liu XB, Muñoz A, Jones EG. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol. 1998; 395: 450-465.
[61] Lourenço Neto F, Schadrack J, Berthele A, Zieglgänsberger W, Tölle TR, Castro-Lopes JM. Differential distribution of metabotropic glutamate receptor subtype mRNAs in the thalamus of the rat. Brain Res. 2000; 854: 93–105.
[62] Shigemoto, R., Nakanishi, S., Mizuno, N. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J. Comp. Neurol. 1992; 322: 121-135.
[63] Stinehelfer S, Vruwink M, Burette A. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 2000; 861: 37–44.
[64] Wijetunge LS, Till SM, Gillingwater TH, Ingham CA, Kind PC. mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. J Neurosci 2008: 28: 13028–13037.
[65] Sun QQ, Zhang Z, Jiao Y, Zhang C, Szab G, Erdelyi F. Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. J Neurophysiol 2009; 101: 2679–2692.
[66] Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD et al. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacol. 2011; 60: 1017-1041.
[67] Ngomba RT, Santolini I, Biagioni F, Molinaro G, Simonyi A, van Rijn CM et al. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacol. 2011; 60: 1281-1291.
[68] D'Amore V, Santolini I, van Rijn CM, Biagioni F, Molinaro G, Prete A et al. Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacol. 2013; 66: 330-338.
[69] Molinaro G, Traficante A, Riozzi B, Di Menna L, Curto M, Pallottino S et al. Activation of mGlu2/3 metabotropic glutamate receptors negatively regulates the stimulation of inositol phospholipid hydrolysis mediated by 5-hydroxytryptamine2A serotonin receptors in the frontal cortex of living mice. Mol Pharmacol. 2009; 76: 379-387.
[70] Vieira E, Huwyler J, Jolidon S, Knoflach F, Mutel V, Wichmann J. Fluorinated 9H-xanthene-9-carboxylic acid oxazol-2-yl-amides as potent, orally available mGlu1 receptor enhancers. Bioorg Med Chem Lett., 2009; 19: 1666–1669.
[71] Rodriguez AL, Grier MD, Jones CK, Herman EJ, Kane AS, Smith RL et al. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and anti-psychotic activity. Mol Pharmacol. 2010; 78: 1105–1123.

[72] Anderson JJ, Rao SP, Rowe B, Giracello DR, Holtz G, Chapman DF et al. [3H]Methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine binding to metabotropic glutamate receptor subtype 5 in rodent brain: in vitro and in vivo characterization. J Pharmacol Exp Ther. 2002; 303: 1044–1051. 18
[73] Cosford ND, Roppe J, Tehrani L, Schweiger EJ, Seiders TJ, Chaudary A et al. [3H]-methoxymethyl-MTEP and [3H]-methoxy-PEPy: potent and selective radioligands for the metabotropic glutamate subtype 5 (mGlu5) receptor. Bioorg Med Chem Lett. 2003; 13: 351–354.
[74] D'Amore V, Santolini I, Celli R, Lionetto L, De Fusco A, Simmaco M et al. Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats. Neuropharmacol. 2014; 85: 91-103.
[75] D'Amore V, von Randow C, Nicoletti F, Ngomba RT, van Luijtelaar G. Anti-absence activity of mGlu1 and mGlu5 receptor enhancers and their interaction with a GABA reuptake inhibitor: Effect of local infusions in the somatosensory cortex and thalamus. Epilepsia. 2015; 56: 1141-1151.
[76] von Krosigk M, Bal T, McCormick DA. Cellular mechanisms of a synchronized oscillation in the thalamus? Science 1993; 261: 361–364.
[77] Porcello, DM, Smith SD, Huguenard JR. Actions of U-92032, a T-type Ca2+ channel antagonist, support a functional linkage between I-T and slow intrathalamic rhythms J. Neurophysiol. 2003; 89: 177–185.
[78] Bandrowski AE, Huguenard JR, Prince DA. Baseline glutamate levels affect group I and II mGluRs in layer V pyramidal neurons of rat sensorimotor cortex. J Neurophysiol. 2003; 89: 1308-1316.
[79] Cope DW, Di Giovanni G, Fyson SJ, Orbán G, Errington AC, Lőrincz ML et al. Enhanced tonic GABAA inhibition in typical absence epilepsy. Nat Med. 2009; 15: 1392–1398.
[80] Lüttjohann A, Zhang S, de Peijper R, van Luijtelaar G. Electrical stimulation of the epileptic focus in absence epileptic WAG/Rij rats: assessment of local and network excitability. Neurosci. 2011; 188: 125-134.
[81] van Luijtelaar G1, Sitnikova E, Luttjohann A.On the origin and suddenness of absences in genetic absence models. Clin EEG Neurosci. 2011; 42 :83-97.
[82] Hosford DA, Lin FH, Wang Y, Caddick SJ, Rees M, Parkinson NJ et al. Studies of the lethargic (lh/lh) mouse model of absence seizures: regulatory mechanisms and identification of the lh gene. Adv Neurol. 1999; 79: 239-252.
[83] Rook JM, Tantawy MT, Ansari MS, Felts AS, Stauffer SS, Emmitte K et al., Relationship between In Vivo Receptor Occupancy and Efficacy of Metabotropic Glutamate Receptor Subtype 5 Allosteric Modulators with Different In Vitro Binding Profiles. Neuropsychopharmacol. 2015; 40: 755–765.
[84] Cleva RM, Olive MF. Positive allosteric modulators of type 5 metabotropic glutamate receptors (mGluR5) and their therapeutic potential for the treatment of CNS disorders. Molecules. 2011; 16: 2097-2106




# 7 The anti-absence effect of mGlu5 receptor amplification with VU0360172 is maintained during and after antiepileptogenesis

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# Abstract

**Purpose:** Ethosuximide (ETX) is the drug of choice for the treatment of patients with absence seizures – taking into account both its efficacy, tolerability and antiepileptogenic properties. However, 47% of subjects failed in ETX-therapy, and most antiepileptic drugs have cognitive side effects. VU0360172, a positive allosteric modulator (PAM) of mGluR5, has been proposed as a new anti-absence drug. Here it is investigated whether anti-epileptogenesis induced by ETX alters the sensitivity of VU0360172, and whether cognition is affected during and after chronic ETX treatment.

**Method:** EEG's were recorded before and after a challenge with VU0360172 in chronic ETX and in control WAG/Rij rats during and after treatment. Rats were also exposed to a cue discrimination learning task in a Y-maze both during and after treatment. At the end of the experiment, mGlu5 receptors were quantified by Western Blot analysis.

**Results:** Antiepileptogenesis was successfully induced by ETX and VU0360172 showed a time and dose dependent anti-absence action in the control group. VU0360172 kept its anti-absence action in chronic ETX treated rats both during and after treatment, without time and dose dependency. This anti-absence effect of VU0360172 in both groups matched the lack of differences in mGluR5 expression. Chronic ETX enhanced the number of completed trials, the number of correct choices in the Y-maze and the number of consumed sucrose pallets.

**Significance:** VU0360172 maintains its anti-absence effects after chronic treatment; as such, VU0360172 can also be used as a adjunctive therapy in patients with absence epilepsy. The enhanced motivation and cognitive performance by ETX might be mediated by the antidepressant action of ETX as expressed by an increase in the rewarding properties of sucrose pallets.


**Key words:** antiepileptogenesis, mGluR5, Electroencephalography, absence epilepsy, WAG/Rij rats, Ethosuximide, Western Blots, Y-maze learning

# Introduction

Childhood absence epilepsy (CAE), a neurological disorder which can be found in about 10% of children with epilepsy, usually occurs around the ages of 4 to 12 years [1]. During an absence, ongoing activity is halted and a person's responsiveness is usually briefly impaired [2]. Furthermore, typical absence epileptic seizures are electrophysiologically characterized by a pattern of bilateral synchronized spike wave discharges (SWDs) [3]. SWDs in the genetic rodent models such as rats of the WAG/Rij strain and GAERS are initiated in the deep layers of the somatosensory cortex and quickly spread to the cortico-thalamo-cortical (C-T-C) network [4,5,6]. Current medical therapies for epilepsy symptomatically suppress seizure activity, but they are not disease modifying, and therefore have no effect on the underlying propensity of the brain to generate seizures. One exception may be that of chronic treatment with ethosuximide (ETX). This may interfere with epileptogenesis, as was suggested by the outcomes of several experimental studies in the genetic models [7,8,9]. A prospective cohort study in children with CAE showed that long-lasting ETX treatment resulted in a higher rate of complete remission, and in more occurrences of five and ten-year remission, suggesting an anti-epileptogenic effect [10].

Evidence has emerged that metabotropic glutamate receptors (mGluR) are good candidates for the treatment of absence epilepsy [11,12,13]. We demonstrated that the potentiation of mGlu5 receptors with the positive allosteric modulator (PAM), VU0360172, reduces SWDs dose dependently and the outcomes of a ten-day chronic administration study showed no loss, or only a very small loss of efficacy with respect to its anti-absence action [14,15]. Next, it was found that VU0360172 reduces the occurrence of SWDs when locally micro-infused in the main areas of the absence network (cortex and thalamus) [16]. This demonstrates that VU0360172 is equally effective in targeting both locations, and this may contribute to successful seizure suppression of mGluR Group I PAMs.

Considering that seizure control cannot often be achieved with monotherapy, drug interaction studies are imperative. The interaction between acutely administrated ETX and valproic acid (VPA) showed infra-additive effects in the WAG/Rij model, suggesting that these two antiepileptic drugs (AED) share a common mode of action [17]. No interactions between acutely administered ETX and non-selective orthosteric agonist/antagonist mGlu5 receptors were found in pentetrazole-induced convulsions in mice [18,19]. However, no drug interaction studies were done on the genetic absence models during chronic ETX treatment aimed at antiepileptogenesis; it was only found that the sensitivity of levetiracetam (LEV) was lost one month following the end of its chronic treatment [20]. Here the (inter)action of VU0360172 with ETX will be investigated in our genetic absence model, both during and after ETX treatment has stopped.

Most AED such as VPA, ETX, and lamotrigine (LTG) have a negative impact on cognition [21,22]. On the other hand, the antiepileptogenic effects of chronic ETX were accompanied by a decrease in depressive-like behavior in WAG/Rij rats [23,24]. Considering

that epileptogenesis and chronic ETX treatment induce antiepileptogenesis, this could have an affect on mood and cognitive processes, such as learning and memory. These latter processes were additionally investigated in a cue discrimination learning task in an Y-maze (problem solving task) and in a sucrose motivation task during and after chronic treatment. This learning task has been found sensitive for the effects of e.g. VPA [25].

The expression of mGlu5 receptors in the thalamus and cortex after chronic treatment with ETX and challenged by VU0360172 was also evaluated in order to investigate whether chronic treatment affects the expression of mGluR5, with respect to changes that could correlate to the temporal profile of the drug's response to the incidence of SWDs.

# Materials and Methods

## Drugs and experimental protocol

ETX (Ethymal 250 mg/4 ml ethosuximide), a blocker of T-type $Ca^{2+}$ currents in thalamic neurons [26], was obtained from Apotex Europe BV, Leiden. VU0360172 (N-clyclobutyl-6-[2-3(fluorophenyl)ethynyl]pyridine-3-carboxamine), a selective mGlu5 receptor PAM, was obtained from Vanderbilt University Medical Center [27]. ETX was administered orally through the drinking water. VU0360172 was dissolved in 10% Tween 80, and injected s.c.

## Animals

The study consisted of 26 male inbred WAG/Rij rats, born and raised at the Donders Centre for Cognition in Nijmegen, The Netherlands. The 26 rats, after weaning, at post-natal day 28 (PND 28), were housed in standard macrolon type III cages in groups of four and five for the first two weeks, then they were housed in pairs. All rats had free access to food and water throughout the whole experiment, and were maintained on a 12:12 h light/dark cycle (lights on at 8:30 am) under controlled conditions (20ºC, 60% humidity). Four rats in this group died during surgery, and the rest of the animals (n=22) were decapitated immediately after the last behavioral test, following the last EEG recording session. All experimental procedures and animal care were carried out in compliance with the Animal Experiments Committee of Radboud University (RU-DEC 2013-269). All efforts were made to minimize animal suffering and to reduce the number of animals used.

## Chronic drug administration protocol and pharmacological challenges with mGluR5 PAM

For the chronic treatment studies, animals from a single litter were divided and allocated as either ETX-treated (n=13) or control (n=13) (tap water)–treated animals in a paired fashion. At four weeks of age when the study began, all animals were able to drink independently, and ETX treatment was initiated in young WAG/Rijs to achieve a palatable

dose of 250 mg/kg/day in the drinking water [7, 24]. Young animals drink more due to fast growth, therefore until day 45 we used 150 mg of ETX per 100 ml. After 45 days, the rate of growth decreased and we used 250 mg ETX per 100 ml water. The doses, including the changes, were identical to those used by other authors [7, 8, 24]. This means that the rats received a dose of 300 mg/kg/day (see Figure 1). Volumes consumed and animal weights were measured daily for the first week, and then weekly thereafter, and the drug dosage administered (mg/kg/day) was recorded. The concentration of drug in the water bottles was updated weekly to maintain the appropriate dosage in an iterative fashion. Control-treated rats received tap water ad libitum for the duration of the study. Due to the light-sensitive nature of ETX, drinking bottles were wrapped with black tape.

To assess the effects of ETX treatment on seizures, the EEG was recorded continuously for 24 hours, two times: once at week 16, and following drug cessation at week 17. EEGs were conducted during treatment at week 16 to check the antiepileptic action of ETX and 1 week post treatment at week 17 to check the putative anti-epileptogenic action of ETX. The drug effects on behavior were determined via Y-maze tests after 6 weeks of treatment, and at weeks 16 and 17 as well. Conducting this at different points in time could help us to discover during which time of treatment they may develop behavioral disturbances. Interestingly, the first week of behavioral tests coincided with the period in which WAG/Rij rats began developing the SWDs (28). In this sense, the rats were at the border of being symptomatic and we were speculating whether the effects of the drug could already be manifesting at this stage. In particular, the reason for scheduling the behavioral tests during weeks 16 and 17 was because we felt it necessary to test the rat's behavior during the end of the chronic treatment, and one week later, when ETX was no longer detectable in the plasma (24). The sucrose motivation tests were scheduled on exactly the same weeks (6, 16 and 17) as the Y-maze tests in order to test our hypothesis that chronic ETX enhances motivation to consume sugar pallets. The outcome of these tests could be considered evidence for the reduction in anhedonia as a consequence of chronic ETX treatment.

A pharmacological challenge with 2 injections of VU0360172, both during treatment and one week after treatment, was performed to establish the efficacy of the compound on seizure activity as measured in the EEG. It is imperative to check the antiepileptic action (week 16: the last week of treatment) of ETX as well as its (ETX) anti-epileptogenic action (week 17).

The pharmacological challenge was scheduled with two single injections of VU0360172 (1 mg/kg, s.c. and 3 mg/kg, s.c.) at 9:00 am and at 11:00 pm, respectively, in both ETX treated and the control group at two different times: during the last few days of the chronic ETX treatment and 6 days after the treatment had stopped. We chose to utilize a more commonly used design, which was a cumulative dose design. In this case, two doses are given on the same day, 2 hours apart, which is sufficient considering our previous pharmacokinetic data. From two of our previous studies, it was concluded that a

dosage of 3 mg/kg was able to reduce SWDs significantly (14,15), and that the effects had disappeared within 2 hours. We wanted to see if a lower dose of VU0360172 would also be effective and/or interact differently than a higher dose did with ETX. Therefore, we also used 1 mg/kg. It was (accurately) expected that the effects of 1 mg/kg (if any) would disappear within 2 hours. Therefore, the second injection was completed 2 hours after the first. In order to ensure that there would be enough time to reach base-line levels after the second injection, we recorded and analyzed 3 more hours of EEGs. Considering that we wanted to compare the same drug both during and after ETX treatment, the above mentioned procedure was also repeated one week later.

The EEGs and the behavior of the rats were recorded (see Figure 1).

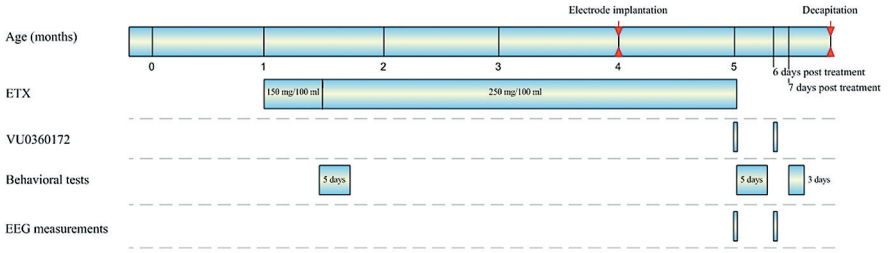


**Figure 1** Schematic diagram illustrating the experimental procedures.

## In vivo recordings; EEG recordings

After 3 months of treatment, at the age of 4 months, the WAG/Rij rats were chronically equipped with a cortical EEG electrode set. A cortical tripolar electrode set (Plastics One®, Roanoke, VA, USA, MS333/1-A) was implanted via stereotactic surgery under isoflurane anesthesia supplemented with pre-and postoperative Rimadyl as analgesic and lidocaïne as local anesthetic.

The first electrode was implanted in the frontal region (coordinates with the skull surface flat and from bregma zero–zero, AP+2, 0: L –3, 5) with a second one in the parietal region (A –6, 0: L –4, 0) [29]. The ground electrode was placed over the cerebellum. After surgery the rats had two weeks to recover, after which, they were moved into transparent EEG recording cages supplied sawdust and cage enrichment and with water and food ad libitum. WAG/Rij rats were connected to an EEG cable with a preamplifier and a swivel, which allowed free movement.

Before recording the rats were habituated to the leads for at least 24h. Each EEG session in order to check the anti-epileptogenic effect induced with ETX lasted 24 h for the treated group and control group as well. The next day after the effects of ETX during

and after treatment were established, the rats were challenged with VU0360172. The EEG session for the pharmacological challenge study with VU0360172 lasted 7h (2 h pre-injection (baseline); 2 h post the first injection; 3 h post the second injection). The differential recorded EEG was filtered (only frequencies between 1 and 100 Hz were allowed to pass) and were digitalized with a sample frequency of 512 Hz, and saved for an off-line analysis using Windaq system (DATAQ, Instruments, Akron, OH, USA). SWDs were labeled visually using common criteria, regular trains of sharp spikes and slow waves lasting from of 1–10 s, spike–wave frequency of 7–10 Hz, a spikes amplitude at least twice the background signal and asymmetric appearance of the SWDs [30,31].

## Spontaneous motor activity

Spontaneous motor activity was recorded as previously reported [32]; an analogic passive infrared detector (PIR) (Luna PR, Rokonet Electronics LTD, Rishon Le Tzion, Israel) was fixed to a semi-open lid on top of the each rat's EEG recording cage. The analogue signal was digitalized simultaneously with the EEG signal. Movements were quantified by calculating the mean of the absolute value of the PIR signal per hour. The values of each individual rat were analyzed to investigate if there were any differences in motor activity between baseline and post injection periods to see if there were any drug effects.

## Western blot analysis of mGlu5 receptors

Twenty two rats were decapitated at the end of the experiment and brains were rapidly removed and frozen. Brains were coded and the codes were only released after the data of the Western blot were secured. Brains were coded and the codes were only released after the data of the Western blot were secured. Brains were cut coronally on a cryostat, and the primary motor cortex (M1), primary somatosensory cortex (S1), reticular thalamic nucleus (RTN) and ventrobasal thalamic nuclei (VB) were manually dissected between bregma -1.88 mm and -3.80 mm under the guide of the Paxinos and Watson atlas (2005). The expression of mGlu5 receptor proteins was estimated by Western blot analysis, using a highly specific polyclonal antibody (1:5000, Abcam, Cambridge, UK) and a mouse monoclonal antibody to label β-actin (1:100.000, Sigma, St. Louis, MO). Anti-mGlu5 receptor antibodies recognized a band of ~130 kDa. Specificity of anti-mGlu5 receptor antibodies was verified using brain protein extracts obtained from mGlu5 receptor knockout mice.

Brain tissues were homogenized at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. After sonication, 2 µl of total extracts were used for protein determinations. One hundred micrograms of protein extract were resuspended in sodium dodecyl sulfate (SDS)-bromophenol blue reducing buffer with 40 mM dithiothreitol (DTT). Western blot analyses were carried out by loading 35 µg of total proteins per lane into 8% SDS polyacrylamide gels, which were electroblotted on immunoblot polyvinylidene difluoride

(PVDF) membranes (BioRad, Milano, Italy). The PVDF membranes were blocked overnight in TBS-T buffer containing 5% non-fat dry milk. Blots were then incubated for 1 h at room temperature with rabbit polyclonal anti-mGlu5 antibodies and mouse monoclonal antibody to label β-actin. Filters were washed with TBS-T buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit or antimouse; 1:7000; Amersham, Piscataway, NJ). Immunoreactivity was revealed by enhanced chemiluminescence (ECL). Immunoreactive protein bands were quantified using the densitometry method (Scion image software, http://rsb.info.nih.gov/nihimage/). Values were obtained by calculating the ratio between the area under the curve (AUC) of the optical density of mGlu5 signal and the AUC of the house keeping protein β-actin for each lane.

## Learning in the Y-maze

Problem solving has been analyzed in a Y-maze [33,34,35,36]. The Y-maze components were a starting box (0.10 m) in a starting arm (0.50 m) which was made with Plexiglas. Two identical arms were placed in a 90 degree angle on the starting arm and another 90 degree angle was made after 0.20 m. At the beginning of the left and right arm the cues (width 0.10 m, length 0.15 m) were presented, Plexiglas and sandpaper [34]. The terminus of the identical arms were 0.20 m and in the termini food trays (0.03 m tall, 0.03 m diameter) were located in which sucrose pallets (Campden, sucrose pallets, 45 mg) could be placed. The Y-maze is 0.10 m in width, 0.10 m high and was covered with Plexiglas.

## Procedure Y-maze

Problem solving was subsequently measured at 6 weeks into treatment, 4 months into treatment and 1 week (for 3 days) after treatment had stopped. First, rats were given a habituation period of 15 minutes 5 days before the beginning testing phase to familiarize them with the Y-maze [33]. 15 pallets were placed in the left and right arm, if the rat ate all the pallets new ones were provided. After the habituation period the rats were randomly split into two groups. The location of the cues for each rat were switched in the different testing phases (example: phase 1 left, phase 2 right, phase 3 left), the cues (whether the sandpaper was associated with food, or Plexiglas) were kept consistent over the different testing phases. The first testing phase consisted of 5 days of testing with 5 learning trials per day per rat. A learning trial took 4 minutes, the rat was placed in the closed starting box, time started running at the moment the rat left the starting box. At the side of the relevant cue 2 sucrose pallets were placed, when the rat ate the sucrose pallets (one or two) he was picked up and placed back into the starting box [34]. The second testing phase (again 5 days and five trials per day) was the same as the first one except a time-out procedure was added after the animal made an incorrect choice. The rats were kept for 10 seconds in the arm of the incorrect cue. The third testing phase was the same as the second but only lasted 3 days. The Y-maze was cleaned after each learning trial with a 70% alcohol solution in water.

The measured variables were the percentage of correct choices and the total number of completed tasks. A task is completed by eating the sucrose pallets, next the rat was placed in the starting box again, until 4 minutes had elapsed. The criterion for making a choice was front paws and the head above/on the cue [37,38].

## Sucrose pallet consumption (SPC) test

The assessment of the SPC test took place in a cage exactly resembling the rat's home cage. A sucrose tray was placed against the wall of a shorter side. Rats were placed on the same location within the cage facing the sucrose tray, which contained a total of 100 sucrose pallets (Campden, 45 mg). They were allowed to explore and eat for a total of 10 minutes after which they were removed and placed back in their home cages. Rats were adapted to the food pallets 2 weeks prior to the test session and not food or water deprived. Dependent variables measured were the number of approaches the rats made towards the sucrose tray and the total amount of sucrose pallets consumed. The SPC test was done 1.5 months of treatment, 4 months of treatment, and 1 week after the discontinuation of treatment.

## Statistical analysis

All statistical procedures were performed using SPSS Version 22.0 (IBM Corp, 2013). EEG recordings lasted 5 hours starting at 9:00 a.m. for establishing the antiepileptic and antiepileptogenic effects of ETX and the vehicle group during and after one week of treatment. The behavioural and EEG part of the study were done with the two groups and the effects of chronic ETX on incidence and mean duration of SWDs, as well as the behavioural activity of animals (PIR) were tested in separate repeated-measures ANOVAs.

The incidence and mean duration of SWDs for the VU0360172 in the water group during and after treatment and for the VU0360172 challenges during and after the chronic ETX treatment was done in four separate repeated measure analysis, in an EEG time-frame of 15 -min epochs. The time of EEG recording (7 h and 10 x 15 minutes blocks post injection) was used as the within-subjects factor. In case of significant main effect (time), a simple contrast analysis was used to isolate differences across time.

Considering that the pre-VU0360172 incidence was rather different for the chronic ETX and water treated group both during and after the chronic treatment, the relative decrease in SWD incidence induced by VU0360172 was compared between the two groups with two repeated measures analyses in the EEG time-frame of 15–min epochs with all values expressed in % of 2 hour pre-injection (baseline), followed by unpaired T-test to establish difference across each time point.

Repeated measures MANOVA were also used to analyze the Y-maze data. This data set was divided across the 3 phases in which Y-maze learning occurred since there were intervals with varying length between the learning sessions. Our within-subjects factor was day (day 1 to 5, day 6 to 10, day 11-13) and the between-subjects factor was drug (ETX,

control). As mentioned, the dependent variables were the percentage correct choices made in the Y-maze and the amount of completed trials.

Unpaired T-tests were used to establish differences in the SPC test, the dependent variable was the number total consumed pallets.

The experimental groups used for the Western blot were the rats chronically treated with ETX + VU0360172 or vehicle + VU0360172. Student's t-test was used for the statistical analysis of each brain region.

# Results

The analysis of the incidence of SWDs in the first 5 h post injection during and after treatment showed that there was no time of day effect, or interaction with time of day with time or group (p= >.05). Instead a very large group effect was present (F= 91.955, df 3, 44 , p< .000, $\eta^2$ =.862). This data confirmed the successful anti-absence and anti-epileptogenic effect induced by ETX without any difference in terms of SWD incidence between the ETX during and/or after the end of treatment. Both ETX groups showed less SWD's compared with the vehicle groups (the data are presented in Figure 2A).

No statistical difference was found in the mean duration of SWDs and in locomotor activity during and after chronic ETX treatment.

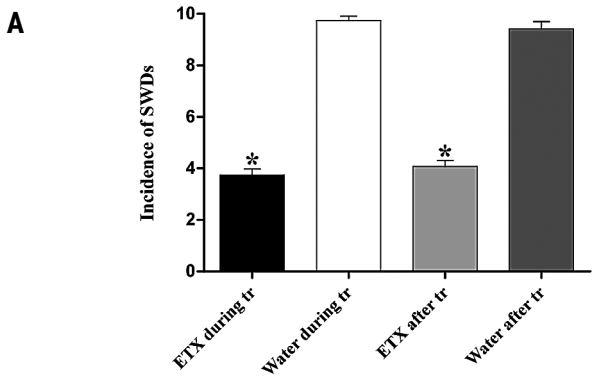


**Figure 2A** Incidence of SWDs during and after ETX treatment (4 months treatment). A significant reduction of the incidence of SWDs was found during (antiepileptic) and one week after the end treatment (antiepileptogenesis). Values are means ± S.E.M of 22 animals. *p< 0.05 (repeated-measures ANOVAs) vs. the corresponding value obtained in control rats.

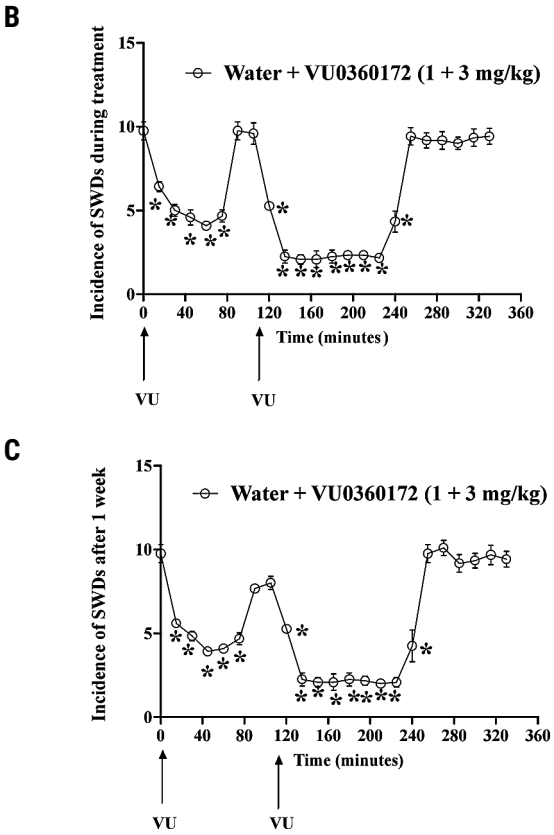


**Figure 2B-C** The effect of two injections of VU0360172 in the water group during and after treatment. Notice the time and dose dependent effects of VU036172 on SWD incidence both during and treatment and after 1 week.

The analysis of incidence of SWD in the water group, during treatment, after the first and second injection of VU0360172 (1/3 mg/kg), revealed a large time effect (blocks) ($F= 54,74$, df 22,242, $p< .001$, $\eta^2 = .83$). A simple contrast analysis revealed that, the water group treated with VU0360172, showed clear time (the low dose of the drug was effective for about one hour, the higher dose for more than 1.5 hr) and dose dependent effects compared to the 2 hr pre-injection control period (the data are presented in Figure 2B).

The same analysis in the water group treated with VU0360172, one week after treatment showed, again a large time effects (block) ($F= 57,28$, df 22,220, $p<.001$, $\eta^2 =.85$); again the simple contrast analysis showed time and dose (the higher dose was longer and more effective) dependent effects (Figure 2C).

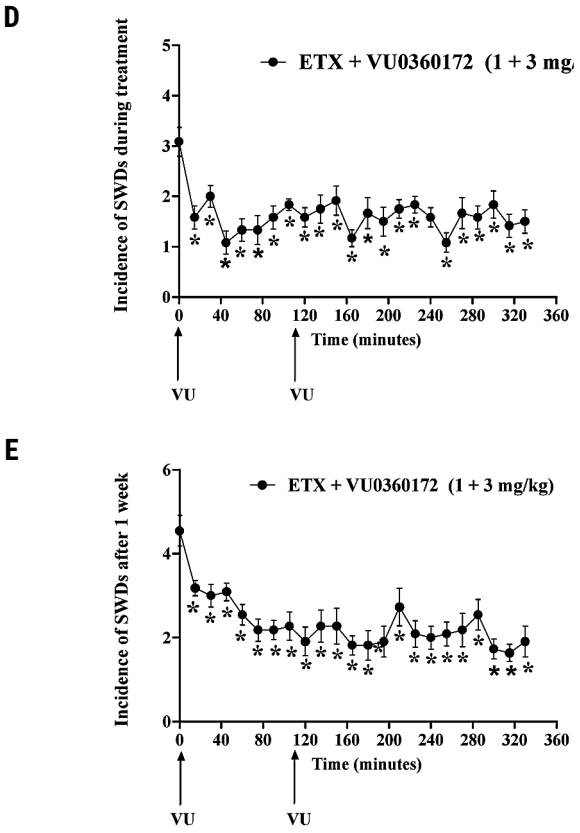


**Figure 2D-E** The pharmacological challenge with VU0360172 (1 mg/kg and 3 mg/kg) with respect the SWD incidence during and after chronic treatment with ETX. Values are means ± S.E.M. of 22 animals. ($p < 0.05$, ANOVA for repeated measures followed by Bonferroni's test) vs. the corresponding values at baseline (T0) (*) or vs.

The analysis of incidence of SWD in the ETX group treated with VU0360172 during treatment showed, a medium sized time effect ($F= 3.07$, df 22,242, $p<.001$, $\eta^2=.22$). A simple contrast analysis showed that all data points indicated the same lower SWD incidence compared to the baseline value (average of 2 hours). This implies a lack of pharmacokinetic effects as were seen in the water + VU0360172 group. The decrease in SWD incidence after VU0360172 administration in the ETX group suggest that the compound still has some effect on SWD incidence, the data are presented in Figure 2D.

Next, the same analyses on the SWD, one week after treatment, showed again a medium sized time effect (blocks) ($F= 3.44$, df 22,220, $p< .001$, $\eta^2 = .26$); again the simple

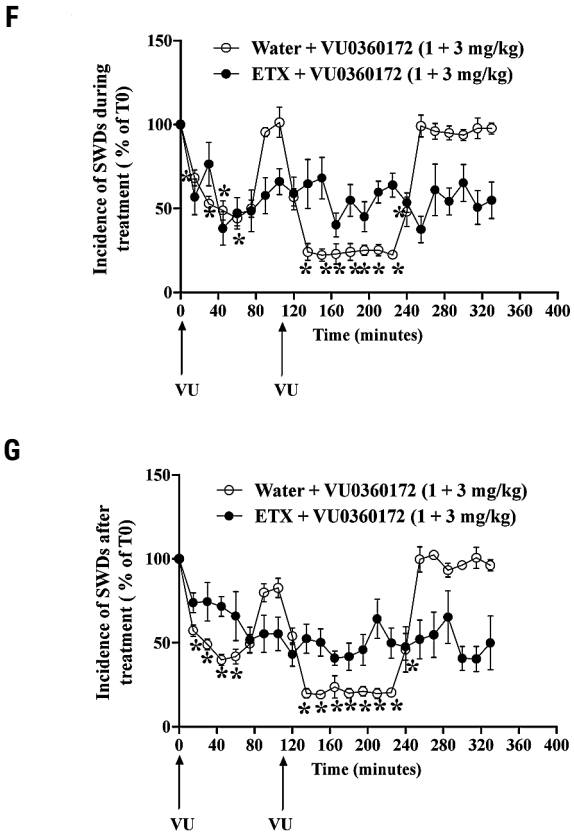


**Figure 2F-G** Comparison of the efficacy of VU0360172 between the chronic ETX and water group (the percentages of the incidence of SWD) after the first and second injection of VU0360172 during treatment and after treatment. Values are means ± S.E.M. of 22 animals. ($p < 0.05$, ANOVA for repeated measures followed by unpaired T-test) vs. the corresponding values at baseline (T0) (*) or vs.

contrast analysis revealed that all data points were lower if compared to the baseline value and again without clear signs of pharmacokinetic and -dynamic effects (Figure 2E). None of the injections with VU0360172 caused significant effects between or within groups in the mean duration of SWDs and in locomotor activity.

The comparison of the efficacy of VU0360172 between the chronic ETX and water group (the percentages of the incidence of SWD) after the first and second injection of VU0360172 (1/3 mg/kg) showed a time effect ($F= 14,12$, df 22,484, $p< .001$, $\eta^2 = .39$), a time x group effect ($F= 10,83$, df 22,484, $p< .001$, $\eta^2 =.33$), while, no group effect was found. This

implies that overall; VU0360172 had the same anti-absence effect in terms of SWD reduction in the ETX and water group. The post-hoc t-tests showed in addition, that the incidence was lower in the control group between 125 and 215 min post injection. At other time points the ETX group was more effective mention the time periods. The data are presented in Figure 2F.

The same analysis of incidence of SWD, one week after treatment, showed again a time effect (F= 18,09, df 22,440, p< .001, $\eta^2$ =.47), a time x group effect (F= 14,08, df 22,440, p< .001, $\eta^2$ =.41), again, no group effect was found. This suggests that over the 6 hrs post drug injection the efficacy of VU0360172 was the same for both groups. Outcomes of post-hoc analyses showed that VU0360172 was more effective in the control versus the chronic ETX group at mention the time episode, at other time points the ETX group was more effective. The data are presented in Figure 2G.

**Expression of mGlu5 receptors in the cortico-thalamo-cortical network of symptomatic WAG/Rij rats after chronic treatment with ethosuximide**

Western blot analysis of mGlu5 receptors was carried out on protein extracts from the motor cortex, somatosensory cortex, RTN and VB of symptomatic WAG/Rij rats after chronic treatment with ethosuximide + VU0360172 or vehicle + VU0360172. Immunoblots showed a major band at 130 kDa corresponding to the mGlu5 receptor monomers. There was no significant difference in mGlu5 receptors expression in cortex and thalamus in symptomatic WAG/Rij rats after chronic treatment with ETX or vehicle (Figure 3).

## Y-maze: Completed trials

***1,5 months into treatment.*** The univariate analysis revealed a day effect (*F*= 12.72, df 2,34, $p < .001$, $\eta^2 = .401$), a drug effect (*F*= 5.85, df 1,19, $p > .026$, $\eta^2 = .235$) and a drug x day interaction (*F*= 5.75, df 2,44, $p > .004$, $\eta^2 = .232$). The total number of completed tasks increased across days, the EXT group completed more tasks and the two groups differed in their changes across days. The ETX group were more eager to complete the task, and showed a bigger improvement over days than the control group (see Figure 4A, block 1).

***4 months into treatment.*** The ANOVA revealed a small day effect (*F*= 3.40, df 2,34, $p < .050$, $\eta^2 = .152$) and a medial strong drug effect (*F*= 6.90, df 1,19, $p < .017$, $\eta^2 = .266$). There was no drug x day interaction. The total completed trials differed across days and the groups differed in the amount of completed tasks: the ETX group performed better than the control group (see Figure 4A, block 2).

***1 week after discontinuation of treatment.*** The univariate analysis revealed no day effect. A drug effect was found (*F*= 13.453, df 1,19, $p < .002$, $\eta^2 = .415$): the ETX group completed more trials than the control group (see Figure 4A, block 3). There was no drug x day interaction.

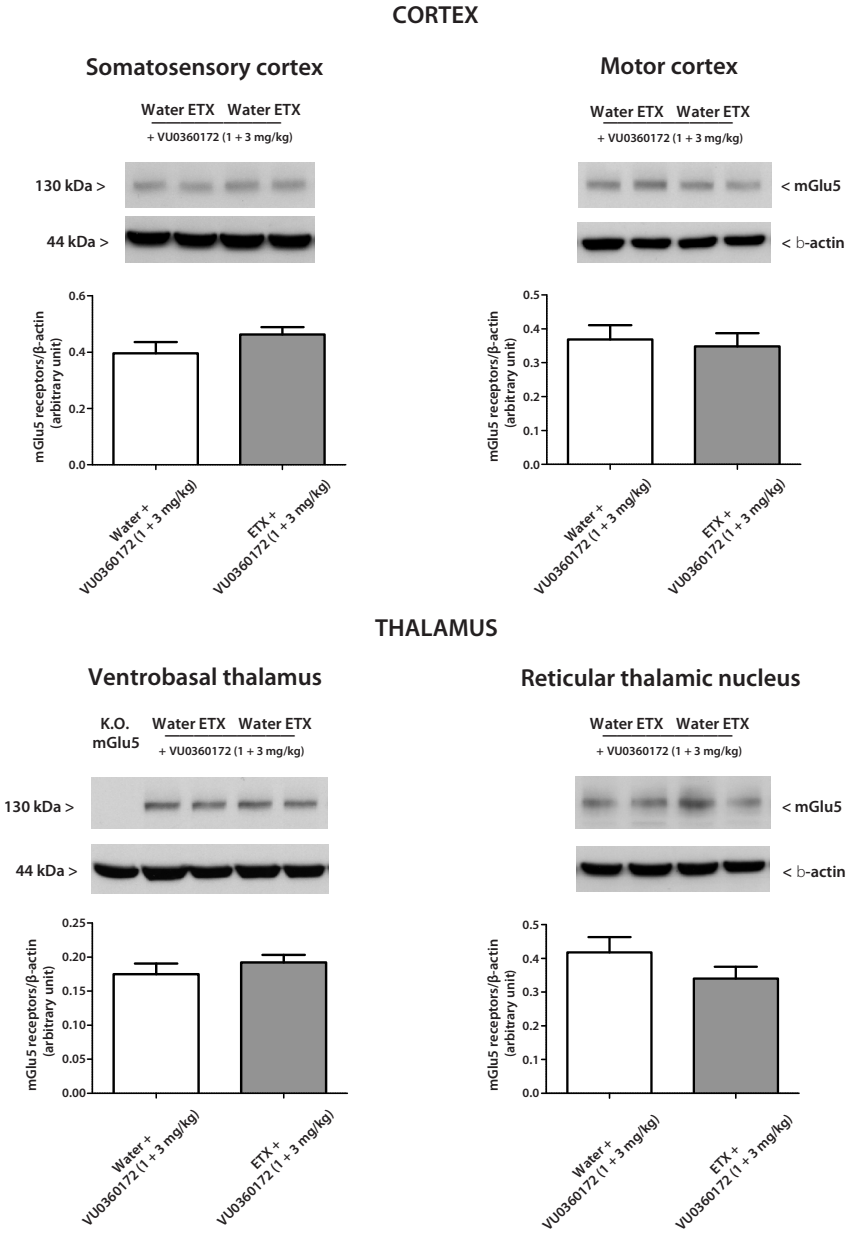


**Figure 3** Immunoblot analysis of mGlu5 receptors in the somatosensory cortex, motor cortex, ventrobasal thalamus and reticular thalamic nucleus of symptomatic WAG/Rij rats after chronic treatment with ethosuximide or vehicle. Densitometric analysis of mGlu5 receptors for each brain region is shown in figure. Values are means ± S.E.M.

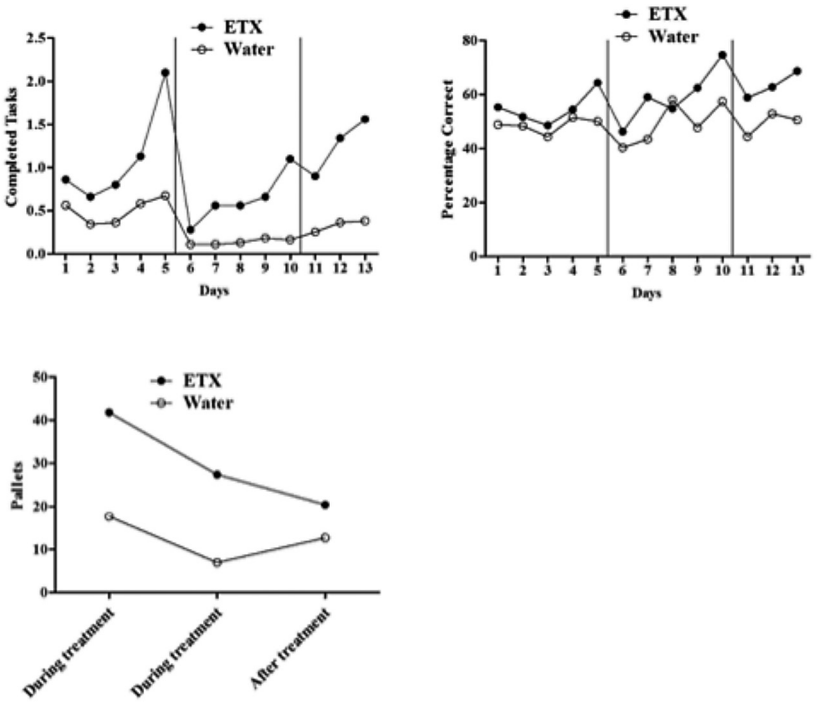


**Figure 4 (Completed task)**: Number (mean) of completed trials in 3 periods (day 1-5 after 1.5 month of treatment; day 6-10 after 4 month of treatment; day 11-13, 1 week after discontinuation of treatment (n = 13) and control (n = 13).
**(Percentage correct)**: Percentage of correct choices (mean) in 3 periods (day 1-5, 1.5 month of treatment, day 6-10, 4 months into treatment, day 11-13, 1 week after discontinuation of treatment), ETX (n = 13) and control (n = 13), with standard error of measurement bars.
**(Pellets)**: Number of consumed pallets (mean) on 3 testing phases (1: 1.5 month of treatment, 2: 4 months into treatment, 3: 1 week after discontinuation of treatment), ETX (n = 13) and control (n = 13).

## Y-maze. Percentage correct

***1,5 months into treatment.*** The ANOVA revealed no day effect, no drug effect, and there was no drug x day interaction. Both groups performed similarly and the total average correct choices did not differ across days (see Figure 4B, block 1).

***4 months into treatment.*** The ANOVA revealed a day effect ($F$= 3.692, df 3,57, $p$< .017, $\eta^2$ = .163). A marginal drug effect was found ($F$= 3.393, df 1,19, $p$< .081, $\eta^2$ = .152). There was no drug x day interaction. In this phase the correct choices differed across days, there is

a trend indicating that the two groups differed in their correct choices but the differences between groups did not differ across days. The data showed that the ETX group performed slightly better than the control group and that the total average correct choices differed across days (see Figure 4B, block 2).

***1 week after discontinuation of treatment.*** The ANOVA revealed a drug effect ($F$= 7.198, df 1,19, $p$< .015, $\eta^2$ = .275), no day effect and no drug x day interaction. The data showed that the ETX group performed better than the control group (see Figure 4B, block 3).

### Sucrose Pallet Consumption (SPC) test

The first SPC test has been done at 1.5 months into treatment. A large and significant effect of drug on the total consumed sucrose intake ($F$= 18.89, df 1,24, $p$< .001, $\eta^2$= .440) has been found: the ETX group consumed more pallets than the control group (see Figure 5).

The SPC test has been done a second time, at 4 months into treatment. The data showed again a drug effect on the total consumed sucrose pallets ($F$= 8.41, df 1,21, $p$< .009, $\eta^2$= .286). The ETX group consumed more pallets than the control group (see Figure 5).

The third SPC test has been done after treatment with ETX had stopped. The analysis revealed a marginal trend for more sucrose pallets in the ETX group ($F$= 3.06, df 1,19, $p$<.096).

## Discussion

The first aim of the study was to investigate if the antiepileptogenic effect induced by ETX and administered via the drinking water altered the sensitivity of group I mGlu receptors. The antiabsence and antiepileptogenic effects were successfully induced by ETX without any differences in terms of SWD occurrence during the treatment, as well as one week after the treatment, as is in line with the outcomes of earlier studies [7,8,9,39,40]. Moreover, chronic ETX treatment has similar disease-modifying effects in GAERS – another genetic model of absence epilepsy [41] .

Our second interest was concerning the interaction between classical ant-epileptic drugs and mGlu modulating compounds with antiepileptic actions. It has been shown that e.g. the non-competitive antagonist mGlu5, SIB 1893, with pro- and anti-convulsant pharmacological activity against electroconvulsive threshold-induced seizures in mice, did not influence the protective action of either ETX, VPA, phenobarbital or clonazepam [19]. Furthermore, the combination of the mGluR5 antagonist with AED did not result in adverse effects [41]. A lack of interaction was also found between a selective Group II agonist, ETX and VPA on PTZ induced convulsions [18]. Here, the sensitivity of group I mGluR in chronic ETX treated rats was evaluated by the administration of VU0360172. As expected [14,15], pharmacological enhancement of mGlu5 receptor activity caused a

robust time and dose-dependent reduction with respect to the incidence of SWD in the control group. This was found two times within a six-day interval, without obvious changes in the sensitivity of the mGlu5 receptor. The effects of the same doses of VU0360172 in the chronic treated ETX rats and water-treated rats during and after treatment also showed the same (percentage-wise) overall anti-absence effects in terms of SWD reduction. This could be clinically relevant because seizure control is not always achieved by a single drug. However, the effects of VU0360172 in the chronic treated ETX rats showed rather striking results: VU0360172 decreased the incidence without any sign of time and dose-dependency. A possible explanation for the lack of dose dependency could be a threshold effect; it might be difficult to reduce an already low number of SWDs. However, the lack of a clear pharmacokinetic effect in the ETX treated rats effect cannot be explained by a threshold effect. In fact, the SWD incidences were reduced by about 40-60% and remained low throughout the entire recording session, without returning to the baseline. It can be speculated that the lack of time dependency may be due to a slowing down of the metabolism of VU0360172 after 4 months of ETX.

The comparable overall efficacy of VU0360172 in the two groups is in agreement with a lack of differentiation in mGlu5R expression between the two groups in the cortex and thalamus. No down- or up-regulation of this receptor was found for the chronic treated group when compared to the age-matched and non-treated control group, suggesting that the system is not modified and VU0360172 does indeed show its anti-absence effect.

Although the overall efficacy of VU0360172 was comparable in the two groups, at many points the efficacy of VU0360172 was higher in the control than in the ETX treated groups. This suggests that chronic ETX influenced the pathways in which VU0360172 exerted its anti-absence action. First of all, as for the ETX mechanisms, a reduction in burst-firing by ETX led to a reduction in SWDs by decreasing the strength of synchronization within the cortico-thalamo-cortical loop during paroxysmal activity [42]. Acute ETX, in addition to its well established success in blocking T-type $Ca^{2+}$ channels, directly reduced excitability by increasing spontaneous GABA release in cortical slices [43]. Moreover, G protein-activated inwardly rectifying $K^+$ channels (GIRK (type GIRK1/2 and GIRK2)) have been shown to play an important role in regulating neuronal excitability, and those channels are activated by various $G_i$ protein-coupled receptors [44,45]. It was found that ETX—but not other antiepileptic drugs—inhibited GIRK1/2 and GIRK2 channels at clinically relevant concentrations in cells of cerebellar slices of mouse brain, suggesting that the inhibitory effects of ETX on GIRK channels may affect the G protein signaling pathways [46]. It is obvious that G-protein signaling pathways are involved in the pathophysiology of absence epilepsy and in SWD control [11,47]. Furthermore, the early and chronic treatment with ETX prevented the commonly reported changes in the expression of $Na^+$ and $HCN^1$ channels in the cortical focal region, as well the local excitability [24,48].

More recent research has been focused on the identification of primary cell signaling

pathways that initially trigger various downstream mechanisms mediating epileptogenesis and, ultimately, a permanent increase in neuronal excitability [49,50]. One signal transduction system that has recently gained interest as an important regulator of cellular changes involved in epileptogenesis is the rapamycin (mTOR) pathway [51,52,53]. In particular, mTOR complex 1 (mTORC1) is involved in a variety of functions and has also been implicated in epileptogenesis (54). Rapamycin had anti-absence properties in acute and sub-chronic treatments of WAG/Rij rats. Conversely, early chronic treatment for 17 weeks had clear antiepileptogeneic effects in the same animal models. These chronic treatment effects could be explained by the ability of rapamycin, through mTOR inhibition, to affect a variety of cellular and molecular processes, such as ion channel expression and neurotransmitter receptors, apoptosis, neurogenesis and synaptic plasticity, all of which are known to influence neuronal excitability and epileptogenesis [55,56,57]. The same kinds of both antiepileptogenic and inhibitions of the mTOR pathway were also found for Vigabatrin (VGB), which is known to cause an increase in GABA levels in the brain and to enhance SWDs in WAG/Rij rats when acutely administered [58]. However, when chronically administered, it had antiepileptogenic effects [38]. VGB inhibited seizures in a mouse model and partially inhibited mTOR pathway activity and glial proliferation in mice, and also reduced mTOR pathway activation in cultured astrocytes from mice [59]. It is known that the mGluR5 agonist, CHPG, activated the mTOR pathway [60]. It is therefore possible to speculate that the PAM VU0360172 also plays a role through the mTOR pathway. This suggests that chronic ETX treatment causes an inhibition in the activity of the mTOR pathway, and VU0360172 may interfere with the mTOR signaling pathway (activation/inhibition) as well. So it could be postulated that VU0360172—although it to some extent maintained its anti-absence effect during and after ETX treatment—lost its common dose-dependency effect due to the already inhibited mTOR signaling by chronic ETX treatment. Further studies into the mTOR pathway after chronic ETX treatment could be a meaningful and interesting follow-up study to elucidate on the involvement of mTOR signaling pathways in anti-absence and epileptogenesis.

The results from the SC test showed that WAG/Rij rats after being treated with ETX for one month are more inclined to consume sucrose pallets than untreated rats, and this was replicated before the end of the chronic treatment. Although this version of sugar consumption has not been validated as a test of anhedonia, a characteristic of depression-like behavior in WAG/Rij rats [61,62], the outcomes do suggest an early anti-anhedonia or antidepressant-like response of ETX already at an early age and early in the treatment regime.

The Y-maze also revealed differences between both groups. Before differences in learning and memory emerged (better cue discrimination was only found at the end of treatment), the treated and untreated groups differed already in the number of completed trials after one month of ETX treatment. At this young age, the treated rats seemed more motivated to run in the Y-maze and they also displayed this above-mentioned increased

interest or appetite for sucrose pallets. Perhaps this caused these rats to be able to learn to discriminate between the cued and non-cued arm of the Y-maze. The positive effects of chronic ETX treatment and the results found were surprising, since most commonly used antiepileptic drugs such as VPA, LTG, and LEV all have a negative impact on cognition [63,34,22,35]. It is proposed that early and chronic ETX treatment has positive motivational effects and this might have facilitated their performance when presented a problem-solving task. Whether the favorable outcome on motivation is due to the effects of ETX perse, or to antiepiletogenesis, still needs to be established. Moreover, it could be possible that the absence of early differences on percentage correct choices in the Y-maze may be explained either by the duration of the treatment (too short) to induce antiepileptogenesis [39], or due to the fact that the number of trials in the learning task was insufficient to detect any effect. The differences found in the test for problem resolution can be attributed to depressive symptoms that were suppressed in the experimental group by ETX. Further research needs to establish whether or not ETX is sufficient enough to reduce depressive-like symptoms in humans as well, in which case additional drug treatments such as anti-depressives should perhaps be considered.

VU0360172 did not affect motor behavior, a beneficial property of an anti-absence drug. Moreover, mGlu5 receptor PAMs are already in development for the treatment of schizophrenia, with the only concern being neurotoxicity and the possibility of convulsive seizures that can be induced by very high doses of these compounds [64]. Additionally, the Phase II clinical trials have now demonstrated promising effects of mGluR5 NAMs in treating anxiety and affective disorders, Parkinsons's disease and fragile X syndrome [65,66], and suggest that the drug can be safely applied.

In conclusion, antiepileptogenesis was successfully induced by ETX, and in the chronically treated rats the anti-absence action of VU0360172, both during and after chronic ETX treatment, was preserved. The lack of pharmacokinetic effects remains enigmatic. The decrease in SWDs during and after ETX treatment was not accompanied by a change in mGluR5 expression. Chronic ETX has minor positive effects on the behavioral performance in the Y-maze, which may be mediated by an increased motivation to obtain sucrose pallets. The combined chronic ETX and acute VU0360172 treatment did not result in any adverse effects, which emphasizes the potential of mGlu receptor ligands to act as adjuncts to current antiepileptic drugs.

## Conflict of Interest Statement

The authors report no potential conflicts of interest.

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# References


[1] Loiseau, P.; Panayiotopoulos, P.; Hirsch, E. Childhood absence epilepsy and related syndromes, in: Roger, J.; Bureau, M.; Dravet, C.; Genton, P.; Tassinari, C.A.; Wolf, P. Epileptic syndroms in infancy, childhood and adolescence. John Libbey & Co Ltd Eastleight, Eastleight 2002, 285-304.

[2] Panayiotopoulos CP. Typical absence seizures and their treatment. Arch Dis Child., 1999, 81, 351-5.

[3] Blumenfeld H. Cellular and network mechanisms of spike-wave seizures Epilepsia 2005, 9, 21-33.

[4] Meeren H.; van Luijtelaar G.; Lopes da Silva F.; Coenen A. Evolving concepts on the pathophysiology of absence seizures: the cortical focus theory. Arch Neurol., 2005, 62, 371-6.

[5] Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S. Deep Layer Somatosensory Cortical Neurons Initiate Spike-and-Wave Discharges in a Genetic Model of Absence Seizures. The Journal of Neuroscience 2007, 27, 6590-6599.

[6] Lüttjohann A.; van Luijtelaar G. The dynamics of cortico-thalamo-cortical interactions at the transition from pre-ictal to ictal LFPs in absence epilepsy. Neurobiol Dis., 2012, 47, 49-60.

[7] Blumenfeld H.; Klein JP.; Schridde U.; Vestal M.; Rice T.; Khera DS.; Bashyal C.; Giblin K.; Paul-Laughinghouse C.; Wang F.; Phadke A.; Mission J.; Agarwal RK.; Englot DJ.; Motelow J.; Nersesyan H.; Waxman SG.; Levin AR. Early treatment suppresses the development of spike-wave epilepsy in a rat model. Epilepsia 2008, 49, 400-9.

[8] Sarkisova KY.; Kuznetsova GD.; Kulikov MA.; van Luijtelaar G. Spike-wave discharges are necessary for the expression of behavioral depression-likesymptoms. Epilepsia 2010, 51, 146-60.

[9] Russo E.; Citraro R.; Scicchitano F.; De Fazio S.; Di Paola ED.; Constanti A.; De Sarro G. Comparison of the antiepileptogenic effects of an early long-termtreatment with ethosuximide or levetiracetam in a genetic animal model of absenceepilepsy. Epilepsia 2010, 51, 1560-9.

[10] Berg AT.; Levy SR.; Testa FM.; Blumenfeld H. Long-term seizure remission inchildhood absence epilepsy: might initial treatment matter? Epilepsia 2014, 55, 551-7.

[11] Alexander GM.; Godwin DW. Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res., 2006, 71, 1-22.

[12] Doherty J.; Dingledine R. The roles of metabotropic glutamate receptors in seizures and epilepsy. Curr Drug Targets CNS Neurol Disord., 2002, 1, 251-60.

[13] Chapman AG.; Yip PK.; Yap JS.; Quinn LP.; Tang E.; Harris JR.; Meldrum BS. Anticonvulsant actions of LY 367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid). Eur J Pharmacol., 1999, 368,17-24.

[14] D'Amore V.; Santolini I.; van Rijn CM.; Biagioni F.; Molinaro G.; Prete A.; Conn PJ.; Lindsley CW.; Zhou Y.; Vinson PN.; Rodriguez AL.; Jones CK.; Stauffer SR.; Nicoletti F.; van Luijtelaar G.; Ngomba RT. Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacology 2013, 66, 330-8.

[15] D'Amore V.; Santolini I.; Celli R.; Lionetto L.; De Fusco A.; Simmaco M.; van Rijn CM.; Vieira E.; Stauffer SR.; Conn PJ.; Bosco P.; Nicoletti F.; van Luijtelaar G.; Ngomba R. Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats. Neuropharmacology 2014, 85, 91-103.

[16] D'Amore V.; von Randow.; Nicoletti F.; Ngomba RT.; van Luijtelaar G. Anti-absence activity of mGlu1 and mGlu5 receptor enhancers and their interaction with a GABA reuptake inhibitor: Effect of local infusions in the somatosensory cortex and thalamus. Epilepsia 2015, 56, 1141-51.

[17] van Rijn CM.; Sun MS, Deckers CL.; Edelbroek PM.; Keyser A.; Renier W.; Meinard. Effects of the combination of valproate and ethosuximide on spike wave discharges in WAG/Rij rats. Epilepsy Res., 2004, 59, 181-9.

[18 ] Kłodzińska A.; Bijak M.; Chojnacka-Wójcik E.; Kroczka B.; Swiader M.; Czuczwar SJ.; Pilc. Roles of group II metabotropic glutamate receptors in modulation of seizure activity. Naunyn Schmiedebergs Arch Pharmacol., 2000, 361, 283-8.

[19] Kinga K.; Borowicz KK.; Piskorska B.; Jarogniew £uszczki.; Stanisaw J.; Czuczwar. Influence of SIB 1893, a selective mGluR5 receptor antagonist, on the anticonvulsant activity of conventional antiepileptic drugs in two models of experimental epilepsy. J. Pharmacol., 2003, 55, 735–740.

[20] Russo E.; Citraro R.; Scicchitano F.; De Fazio S.; Di Paola ED.; Constanti A.; De Sarro G. Comparison of the antiepileptogenic effects of an early long-term treatment with ethosuximide or levetiracetam in a genetic animal model of absence epilepsy. Epilepsia 2010, 51, 1560-9.

[21] Conant, L.; L., Wilfong, A.; Inglese, C. & Schwarte, A. Dysfunction of executive and related processes in childhood absence epilepsy. Epilepsy & Behavior 2010, 18, 414-423.

[22] Pavone P.; Bianchini R.; Trifiletti RR.; Incorpora G.; Pavone A.; Parano E. Neuropsychological assessment in children with absence epilepsy. Neurology 2001, 56,1047-51.

[23] Sarkisova, K.Y.; Kuznetsova, G.D.; Kulikov, M.A.; & van Luijtelaar, G. Spike-wave discharges are necessary for the expression of behavioral depression-like symptoms. Epilepsia 2010, 51, 146-160.

[24] van Luijtelaar .;, Mishra AM.; Edelbroek P.; Coman D.; Frankenmolen N.; Schaapsmeerders P.; Covolato G.; Danielson N.; Niermann H.; Janeczko K.; Kiemeneij A.; Burinov J.; Bashyal C.; Coquillette M.; Lüttjohann A.; Hyder F.; Blumenfeld H.; van Rijn CM. Anti-epileptogenesis: Electrophysiology, diffusion tensor imaging and behavior in a genetic absence model. Neurobiol Dis., 2013, 60,126-38.

[25] Luszczki JJ.; Wojcik-Cwikla J.; Andres MM.; Czuczwar SJ. Pharmacological and behavioral characteristics of interactions between vigabatrin and conventional antiepileptic drugs in pentylenetetrazole-induced seizures in mice: an isobolographic analysis. Neuropsychopharmacology 2005, 30, 958-73.

[26] Coulter DA.; Huguenard JR.; Prince DA. Characterization of ethosuximide reduction of low-threshold calcium current in thalamic neurons. Ann Neurol., 1989, 25, 582-593.

[27] Williams, R.; Manka, J.T.; Rodriguez, A.L.; Vinson, P.N.; Niswender, C.M.; Weaver, C.D. Synthesis and SAR of centrally active mGlu5 positive allosteric modulators based on an aryl acetylenic bicyclic lactam scaffold. Bioorg. Med. Chem. Lett., 2011, 21, 1350–1353.

[28] The Rat Brain in Stereotaxic Coordinates. Front Cover. George Paxinos; Charles Watson. Elsevier Academic Press, 2005

[29] van Luijtelaar, E.; L., & Coenen, A. M. Two types of electrocortical paroxysms in an inbred strain of rats. Neuroscience Letters 1986, 70, 393-397.

[30] Ovchinnikov A.; Lüttjohann A.; Hramov A.; van Luijtelaar G. An algorithm for real-time detection of spike-wave discharges in rodents. J Neurosci Methods., 2010, 194, 172-8.

[31] van Rijn CM.; Gaetani S.; Santolini I.; Badura A.; Gabova A.; Fu J.; Watanabe M.; Cuomo V.; van Luijtelaar G.; Nicoletti F.; Ngomba RT. WAG/Rij rats show a reduced expression of $CB_1$ receptors in thalamic nuclei and respond to the CB receptor agonist, R(+)WIN55,212-2, with a reduced incidence of spike-wave discharges. Epilepsia 2010, 51,1511-21.

[32] Conrad, C. D.; Grote, K. A.; Hobbs, R. J.; & Ferayorni, A.; Sex differences in spatial and non-spatial Y-maze performance after chronic stress. Neurobiology of Learning and Memory 2003, 79, 32-40.

[33] Hidaka, N;, Suemaru, K.; Takechi, K.; Li, B. & Araki,; H. Inhibitory effects of valproate on impairment of Y-maze alternation behavior induced by repeated electroconvulsive seizures and c-Fos protein levels in rat brains. Acta MedOkayama 2001, 65,269-277.

[34] Diez-Chamizo; V., Sterio.; D., & Mackintosh.; N. J. Blocking and overshadowing between intra-maze and extra-maze cues: A test of the independence of locale and guidance learning. The Quarterly Journal of Experimental Psychology 1985, 37, 235-253.

[35] Thompson, R.; Crinella, F.; M., & Yu. JBrain mechanisms in problem solving and intelligence: A lesion survey of the rat brain. New York: Plenum Press. 1990

[36] Jones, N. C.; Salzberg, M. R.; Kumar, G.; Couper, A.; Morris, M. J. & O'Brien, T. J. Elevated anxiety and depressive-like behavior in a rat model of genetic generalized epilepsy suggesting common causation. Experimental neurology 2008, 209, 254-260.

[37] Wright, R. L.; & Conrad, C. D. (2005). Chronic stress leaves novelty-seeking behavior intact while impairing spatial recognition memory in the Y-maze. Stress 2005, 8, 151-154.

[38] Russo E.; Citraro R.; Scicchitano F.; Urzino A.; Marra R.; Rispoli V.; De Sarro G. Vigabatrin has antiepileptogenic and antidepressant effects in an animal model of epilepsy and depression comorbidity. Behav Brain Res., 2011 225, 373-6.

[39] van Luijtelaar G.; Mishra AM.; Edelbroek P.; Coman D.; Frankenmolen N.; Schaapsmeerders P. Anti-epileptogenesis: Electrophysiology, diffusion tensor imaging and behavior in a genetic absence model. Neurobiol Dis., 2013, 60, 126-138.

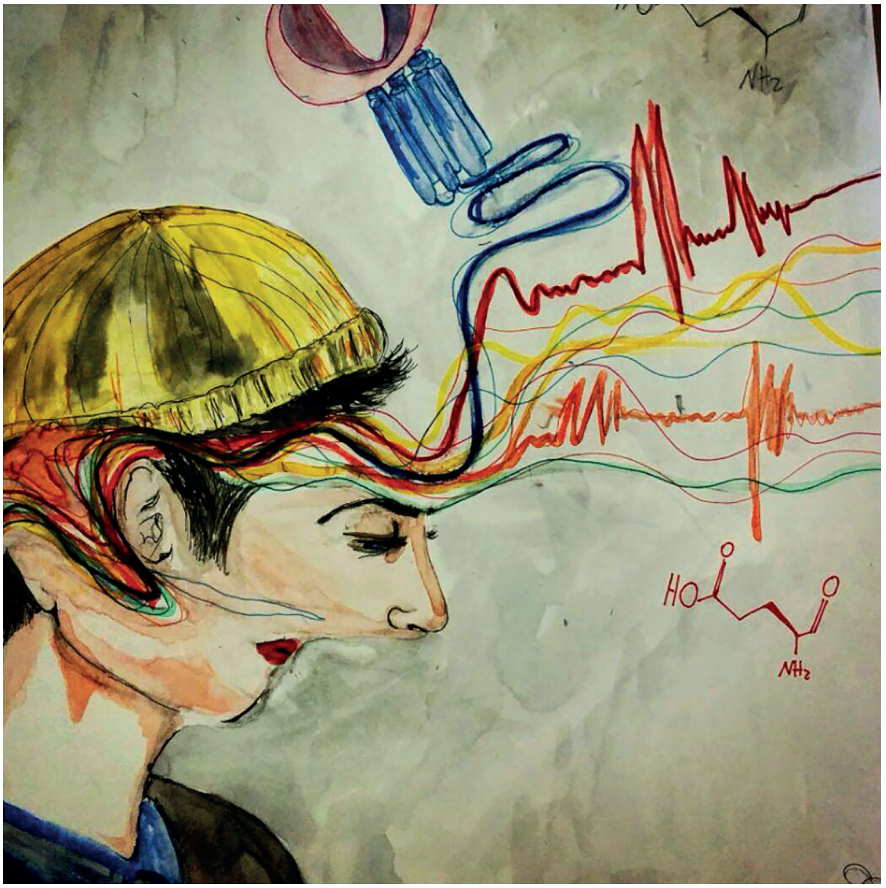
[40] Dezsi G.; Ozturk E.; Stanic D.; Powell KL.; Blumenfeld H.; O'Brien TJ.; Jones NC. Ethosuximide reduces epileptogenesis and behavioral comorbidity in the GAERS model of genetic generalized epilepsy. Epilepsia 2013, 54, 635-43.
[41] Borowicz KK.; Piskorska B.; Łuszczki J.; Czuczwar SJ. Influence of SIB 1893, a selective mGluR5 receptor antagonist, on the anticonvulsant activity of conventional antiepileptic drugs in two models of experimental epilepsy. Pol J Pharmacol., 2003, 55, 735-40.
[42] Leresche N.; Parri HR, Erdemli G.; Guyon A.; Turner JP.; Williams SR.; Asprodini E.; Crunelli V. On the action of the anti-absence drug ethosuximide in the rat and cat thalamus. J Neurosci., 1998, 18, 4842-53.
[43] Greenhill SD.; Morgan NH.; Massey PV.; Woodhall GL.; Jones RS. Ethosuximide modifies network excitability in the rat entorhinal cortex via an increase in GABA release. Neuropharmacology 2012, 62, 807-14.
[44] Dascal, N. Signaling via the G-protein-activated K+ channel. Cell Signal 1997, 9, 551-573.
[45] Kobayasi, T.; Ikeda, K. G protein activated inwardly rectifying potassium channels as potential therapeutic targets. Curr. Pharm. Des., 2006, 12, 4513-4523.
[46] Kobayashi T.; Hirai H.; Iino M.; Fuse I.; Mitsumura K,; Washiyama K.; Kasai S.; Ikeda K. Inhibitory effects of the antiepileptic drug ethosuximide on G protein-activated inwardly rectifying K+ channels. Neuropharmacology 2009, 56,499-506.
[47] Ngomba RT.; Santolini.,, Salt TE.; Ferraguti F.; Battaglia G.; Nicoletti F.; vanLuijtelaar G. Metabotropic glutamate receptors in the thalamocortical network:strategic targets for the treatment of absence epilepsy. Epilepsia 2011, 52, 1211-22.
[48] Blumenfeld H.; Klein JP.; Schridde U.; Vestal M.; Rice T.; Khera DS.; Bashyal C.; Giblin K.; Paul-Laughinghouse C.; Wang F.; Phadke A.; Mission J.; Agarwal RK.; Englot DJ.; Motelow J.; Nersesyan H.; Waxman SG.; Levin AR. Early treatment suppresses the development of spike-wave epilepsy in a rat model. Epilepsia 2008, 49, 400-9.
[49] Dichter MA. Models of epileptogenesis in adult animals available for antiepileptogenesis drug screening. Epilepsy Res., 2006, 68, 31-5.
[50] Pitkänen A.; Lukasiuk K. Molecular biomarkers of epileptogenesis. Biomark Med., 2011, 5,629-33.
[51] McDaniel SS.; Wong M. Therapeutic role of mammalian target of rapamycin (mTOR) inhibition in preventing epileptogenesis. Neurosci Lett., 2011, 497, 231-9.
[52] Russo E.; Citraro R.; Donato.; Camastra C.; Iuliano R.; Cuzzocrea S, Constanti A.; De Sarro G. mTOR inhibition modulates epileptogenesis, seizures and depressive behavior in a genetic rat model of absence epilepsy. Neuropharmacology 2013, 69, 25-36.
[53] Zeng LH.; Rensing NR.; Wong M. The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. J Neurosci., 2009, 29, 6964-72.
[54] Galanopoulou AS.; Gorter JA.; Cepeda C. Finding a better drug for epilepsy: the mTOR pathway as an antiepileptogenic target. Epilepsia. 2012, 53, 1119-30.
[55] Tang SJ.; Reis G.; Kang H.; Gingras AC.; Sonenberg N.; Schuman EM. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc Natl Acad Sci U S A 2002, 99, 467-72.
[56] Kumar V.; Zhang MX.; Swank MW.; Kunz J.; Wu GY. Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci., 2005, 25, 11288-99.
[57] Wang X.; Sun DF.; Fang JY. Research advances on the relationship of PI3-kinase/Akt/mTOR pathway and epigenetic modification. Yi Chuan 2006, 28, 1585-90.
[58] Bouwman BM.; van Lier H.; Nitert HE.; Drinkenburg WH.; Coenen AM.; van Rijn CM. The relationship between hippocampal EEG theta activity and locomotor behaviour in freely moving rats: effects of vigabatrin. Brain Res Bull., 2005, 64, 505-9.
[59] Zhang B.; McDaniel SS.; Rensing NR.; Wong M. Vigabatrin inhibits seizures and mTOR pathway activation in a mouse model of tuberous sclerosis complex. PLoS One 2013, 8, e57445.
[60] Cao L.; Tian Y.; Jiang.; Zhang GJ.; Lei H.; Di ZL. Down-regulation of Homer1b/c protects against chemically induced seizures through inhibition of mTOR signaling. Cell Physiol Biochem., 2015, 35, 1633-42.
[61] Sarkisova KY.; Midzianovskaia IS.; Kulikov MA. Depressive-like behavioral alterations and c-fos expression in the dopaminergic brain regions in WAG/Rij rats with genetic absence epilepsy. Behav Brain Res., 2003, 144, 211-26.
[62] Sarkisova K.; van Luijtelaar G. The WAG/Rij strain: a genetic animal model of absence epilepsy with comorbidity of depression. Prog Neuropsychopharmacol Biol Psychiatry 2011, 35, 854-76.

[63] Conant L.; Wilfong A.; Inglese C.; Schwarte A. Dysfunction of executive and related processes in childhood absence epilepsy. Epilepsy Behav., 2010, 18, 414-23.

[64] Parmentier-Batteur S.; Hutson PH.; Menzel K.; Uslaner J.; Mattson BA.; O'Brien JA.; Magliaro BC.; Forest T.; Stump CA.; Tynebor RM.; Anthony NJ.; Tucker TJ.; Zhang XF.; Gomez R.; Huszar SL.; Lambeng N.; Fauré .; Le Poul E.; Poli S.; Rosahl T.; Rocher JP. Hargreaves R.; Williams TM.; Mechanism based neurotoxicity of mGlu5 positive allosteric modulators--development challenges for a promising novel antipsychotic target. Neuropharmacology 2014, 82, 161-73.

[65] Emmitte KA. mGlu5 negative allosteric modulators: a patent review (2010-2012). Expert Opin Ther Pat., 2013, 23, 393-408.

[66] Rocher JP.; Bonnet B.; Boléa C.; Lütjens R.; Le Poul E.; Poli S.; Epping-Jordan M.; Bessis AS.; Ludwig B.; Mutel V. mGluR5 negative allosteric modulators overview: a medicinal chemistry approach towards a series of novel therapeutic agents. Curr Top Med Chem., 2011, 11,680-95.




# 8 General discussion

# General discussion

There is presently sufficient amount of evidence supporting our understanding of individual roles of mGlu receptor subtypes in the control of SWDs elicited in the cortico-thalamo-cortical (C-T-C) network. As discussed in Chapter 1, many of the ionotropic glutamate receptors agonists/antagonists had toxic effects and their function in seizure control has not led to the successful development of new antiabsence or anticonvulsant drugs (Bruno et al., 2001; Barton et al., 2003; Alexander et al., 2006). There is however extensive support for the statement that we are starting to gain significant knowledge about the individual roles of mGlu receptors in the control of SWDs elicited in the cortico-thalamo-cortical (C-T-C) network. Remarkably, the modulatory role of individual mGlu receptor subtypes on excitatory and inhibitory synaptic transmission in the C-T-C network makes subtype-selective mGlu receptor ligands possible candidates as novel antiabsence drugs. Evidence of phase I and phase II clinical trials with mGlu2/3, mGlu4, or mGlu5 receptor compounds support that these types of ligands might be securely given to humans. This thesis aims to investigate the pharmacological effects of group I mGlu receptor modulation (mGluR1 and mGluR5) in an animal model of absence epilepsy, WAG/Rij rats, their role in SWD occurrence in cortex and thalamus, and their interactions with the clinically commonly used most AED ethosuximide chronically administered.

## The role of mGluR1 and mGluR5 in SWD activity

In Chapter 2, the function, expression, and pharmacological manipulation of mGlu1 receptors was examined in WAG/Rij rats. The main result was that pharmacological manipulation with the mGluR1 PAM, RO0711401 (3, 10 and 30 mg/kg), showed a dose-dependent reduction in the number and mean duration of SWDs. Treatment with the selective mGlu1 receptor NAM, JNJ16259685 (2.5 and 5 mg/kg), enhanced the number of SWDs in a dose dependent way. Moreover, RO0711401 slightly increased spontaneous motor activity.

The results of the experiment investigating mGlu1 receptor signaling displayed a decrease in the function of this receptor in the thalamus of symptomatic WAG/Rij rats compared to age-matched control ACI rats. This decreased mGlu1 function in the thalamus is in agreement with the outcomes that the deletion of phospholipase-C β4 (PLCβ4) in thalamic relay nuclei leads to absence seizures (Cheong et al., 2009). PLCβ4 is an enzyme activated by mGlu1 receptors and is found in thalamo-cortical neurons, where it functions as downstream signaling for the mGlu1 receptor to mediate cortico-thalamic excitatory inputs. The expression of PLCβ4 corresponds to that of the mGlu1 receptors (Watanabe et al., 1998; Miyata et al., 2003). Thereby, interruption of the group 1 signaling pathway (i.e. deletion of PLCβ4 in thalamus) causes SWDs. The reduction in mGlu1 receptor expression that we found did occur in the VPM and PO, areas involved in the SWD network (Lüttjohann and van Luijtelaar, 2012). As discussed in Chapter 1, SWD occur

in the peri-oral region of the somatosensory cortex (Meeren et al., 2002), and the comparable somatotopic region in the thalamus is the lateral region of the thalamus (Polack et al., 2009). Therefore, we proposed that in WAG/Rij rats a reduced excitation in relay neurons due to a decrease in mGlu1 receptor expression found in the VPM and PO might be also responsible for the SWD occurrence.

Pharmacological manipulation of mGlu1 receptors had a robust influence on SWD in WAG/Rij rats. Interestingly, mGlu1 and mGlu5 receptors have a different distribution pattern in the C-T-C circuitry, which suggests distinct rather than complementary roles of these two receptor subtypes. Therefore, in order to have a more detailed understanding of both group I receptors in this absence model, the role of mGlu5 receptors was addressed in Chapter 3. Pharmacological potentiation of this receptor with VU0360172 (3 and 10 mg/kg) decreased numbers and mean durations of SWDs, without affecting motor behavior. The results of pharmacological blockade of mGlu5 receptors with the antagonist MTEP (10 and 30 mg/kg) did not influence SWDs. In order to prove that mGlu5 PAM is selective for this receptor, VU0360172 was injected at a dosage of 3 mg/kg in WAG/Rij rats, pre-treated with the selective antagonist MTEP (10 and 30 mg/kg). The effect of VU0360172 was successfully antagonized by MTEP, demonstrating that the drug reduced absence seizures by amplifying the endogenous activation of mGlu5 receptors. A decrease in both the mGlu5 receptor protein levels and mGlu5-receptor function in the thalamus was found when compared to the age matched non-epileptic ACI rats. Conversely, the expression of mGlu5 receptors was enhanced in the motor cortex and in the somatosensory cortex without an accompanying change in mGlu5-receptor function when compared to ACI rats as well. Interestingly, these changes in the expression come first the onset of the epileptic phenotype, because they were previously visible in pre-symptomatic WAG/Rij rats when compared to the ACI rats. Consequently, the mGlu5 receptor expression in the cortex and thalamus is preceding the origin of SWDs and, for this reason, is not dependent of seizure activity. This is weird because modification in the mGlu1 receptors expression in the thalamus arises in symptomatic WAG/Rij rats (see Chapter 2). All in all, adaptive changes regarding the mGlu5 receptors might lay at the core of the absence-seizure prone phenotype of WAG/Rij rats, and mGlu5 receptor enhancers could be develop for the treatment of absence epilepsy.

## Benefits of the acute studies

The consistent outcomes of the pharmacological studies as described in Chapters 2 and 3 permitted us to cover the until now missed role of group I PAM in non-convulsive epilepsy (see Chapter 1, pg. 36, Table 4). Now, a complete overview of both mGlu1-mGlu5 agonists/antagonist and PAMs/NAMs in convulsive and non-convulsive epilepsy can be given (Table 5). The direction of the effects of group I PAM/NAM in terms of increase/decrease of absence epilepsy was predicted based on the available data as were presented in Table 3: where, orthosteric agonist of group I were pro-convulsive agents, while all orthosteric

antagonist were both anti-convulsive as well as anti-absence drugs. Thus, a group I NAM should decrease absence seizures and a group I PAM should increase absence seizures. A complete opposite effect emerged regarding the direction of the effects. As described in Chapters 2 and 3, the mGlu1 NAM, JNJ16259685, increased SWDs. The increase in SWDs may be due to mGlu1 receptors being hypofunctional in the thalamus. Nevertheless, the same NAM, JNJ16259685, had no effect in terms of SWD reduction/ enhancement in non-epileptic ACI rats. The unsuccessful mGlu1 receptor blockage in ACI rats indicates that a decreased function of mGlu1 receptors is not enough to originate absence seizures, and that an exact genotype is needed to generate the effects.

Thereby, according to Table 5, at least 2 different conclusions might be extrapolated from the data obtained from Chapter 2 and 3 regarding group I PAMs/NAMs:

- Orthosteric antagonists are good anticonvulsant agents, but in non-convulsive absence epilepsy, PAM's are suited for blocking SWDs.
- Orthosteric agonists are proconvulsant, while NAMs have either no effect of enhance non-convulsive absence seizures.

The differences regarding how group I drugs react towards convulsive epilepsy on the one hand and non-convulsive epilepsy on the other, is reminiscent to the effects found with GABA mimetics. They are generally very efficient regarding convulsive epilepsy, but they aggravate absence epilepsy (Coenen and van Luijtelaar, 2003). This may suggest that our PAMS and perhaps also our NAM (JNJ162599685), in vivo, are activating GABA receptors or blocking GABA receptors respectively.

**mGlu compounds in two absence models: lh/lh mice vs. WAG/Rij rats**

As described in Chapter 2, the hypothesis that an impaired mGlu1 receptor signaling in the thalamus and systemic administration of a group I NAM facilitates the occurrence of SWDs is not in agreement with data found in lethargic mice (see Table 4, Table 5, and Table 6), since treatment with orthosteric mGlu1 receptor antagonists reduced the occurrence of SWDs in these mice (Chapman et al., 1999). Also the results, as described in Chapter 3, that pharmacological potentiation of mGlu5 receptor provoked a sturdy dose-dependent decrease in the number and mean duration of SWDs in WAG/Rij rats, while the antagonist of this group MTEP did not increase/decrease SWDs (Table 6), is opposite to what was reported in lethargic mice.

A deducible outcome might be that the function of mGlu1 and mGlu5 receptors in the two genetic absence models is not similar, and the role of mGlu5 receptor PAMs/ NAMs is dependent on the genetic background of the animals. However, there are obvious differences between the two models. First of all, lh/lh mice are monogenic models when compared to WAG/Rij rats, which are polygenic models. Secondly, lh/lh

**Table 5** Overview of group I mGlu receptors in convulsive and non-convulsive epilepsy. [1]Moldrich et al., 2003; [2]Shannon et al., 2005; [3]Chapman et al., 1999; [4]Ngomba et al., 2012; [5]D'Amore et al., 2013.

| | CONVULSIVE EPILEPSY<br>Orthosteric Agonist | CONVULSIVE EPILEPSY<br>Orthosteric Antagonist | NON-CONVULSIVE EPILEPSY WAG/Rij rats<br>PAM | NON-CONVULSIVE EPILEPSY WAG/Rij rats<br>NAM |
|---|---|---|---|---|
| mGlu1 | [1]DHPG ↑<br>[1]Quisqualate ↑ | [1]AIDA ↓<br>[1]LY367385 ↓<br>[2]LY456236 | [4]RO0711401 ↓ | [4]JNJ16259685 ↑ |
| mGlu5 | [1]CHPG ↑<br>[1]Quisqualate ↑ | [3]SIB 1893 ↓ | [5]VU0360172 ↓ | [5]MTEP (no effect on SWDs) |

mice, develop ataxia when the cerebellum is also involved, motor disturbances are lacking in the WAG/Rij model. Thirdly, as is shown in Table 6, the same compound LY379268, has been tested in lh/lh mice and WAG/Rij rats with opposite effects in both models. LY379268 reduced absence epilepsy in lh/lh mice, while enhanced absence epilepsy in WAG/Rij rat, suggesting that the two animals model are really different regarding the role of group II mGluR in absence epilepsy. Fourthly, the cortex and thalamus could play a different role in each of these absence models. In lh/lh mice the ventrolateral and nRT are the dynamo networks of the seizures (Hosford et al., 1995), whereas in WAG/Rij rats, SWDs origin is in the peri-oral region of the somatosensory cortex and the nRT has a resonance function (Meeren et al., 2002; Lüttjohann and van Luijtelaar, 2015). In WAG/Rij rats mGlu5 receptors in the thalamus are hypofunctional, and neither this nor the cortical focal origin is investigated in lh/lh mice. Sixthly: as was previously discussed, a functional coupling of mGlu1 receptors and T-type VSCCs (Hildebrand et al., 2007, 2009) could be implicated in the control of SWDs by mGlu1 receptors. lh/lh mice are characterized by a mutation in the β4 subunit of VSCCs and the above mentioned coupling of mGluR1 and T-type VSCCs may be affected and this might be a reason for the results at variance with those found in WAG/Rij rats (Burgess et al., 1999). Whether this is also the case in WAG/Rij rats is not known.

**Table 6** [1]Burgess et al. (1997); [2]Chapman et al. (1999); [3]Chapman et al. (2000); [4]Lojkova´ and Mares (2005); [5]Cheong et al. (2009); [6]Ngomba et al. (2005); [7]Moldrich et al. (2001); [8]Ngomba et al. (2008); [9]Ngomba et al. (2011); [10]D'Amore et al. (2013).

| | Ih/Ih mice | | WAG/Rij rat | | | |
|---|---|---|---|---|---|---|
| | Orthosteric Agonist | Orthosteric Antagonist | Orthosteric Agonist | Orthosteric Antagonist | PAM | NAM |
| Group I | n.a | [1] AIDA<br>[2]LY367385<br>[3-4] MPEP (mGlu5 NAM/ weak mGlu4 PAM)<br>[5] Deletion of PLCβ4 in the thalamocortical relay nuclei leads to absence seizure ↓ | n.a | n.a | [9] RO0711401<br>[10]VU035017210 ↓ | [6]JNJ162596 85 ↑<br>[10] MTEP (no effect on SWDs) |
| Group II | [7] LY379268 ↓ | n.a | [8]LY379268 ↑ | [6] LY341495 ↑ | n.a | n.a |
| Group III | n.a | n.a | [9] PHCC ↑ | n.a | n.a | n.a |

All these factors may contribute to an explanation for the different effects seen in these two absence models, with respect to the direction of the pharmacological effects with mGluR agonist and antagonist and modulators. A comparison between the two absence models with the same PAMs and NAMS should solve the issue whether the opposite outcomes as obtained in the two models are due to differences between an orthosteric agonist and a PAM (or orthosteric antagonist and a NAM) or to differences between the two absence models. The available evidence goes in the direction of differences between the two models.

## Does tolerance develop against group I PAMs?

The outcomes of the acute pharmacological experiments (Chapters 2 and 3) with RO0711401 and VU0360172, respectively have potential relevance from a therapeutic standpoint since antiepileptic drugs will be chronically administered. In Chapter 4 the effects of mGlu1 and mGlu5 PAMs during chronic treatment were investigated. Rats were treated with an effective dose of RO0711401 (10 mg/kg) or VU0360172 (3 mg/kg) twice daily for ten days, and then after 3 days of withdrawal in response to the drug, re-challenge with VU0360172 or RO0711401 respectively. Complete tolerance developed to RO0711401 on the third day of treatment and the rats were still refractory to the drug two days after

treatment withdrawal, whereas VU0360172 maintained its anti-absence activity during and after the treatment. In order to explain why tolerance developed to RO0711401 pharmacokinetic data of the two drugs were collected: VU0360172 has high plasma-protein binding and small first-pass metabolism (Rodriguez et al., 2010). R00711401 shows a little elimination half-life (<2 h), although we found that the effects in terms of SWD reduction lasted 6 hours, it might be due to some active metabolite that are still able to reduce SWDs. However, no information is available on liver metabolism (Vieira et al., 2009). It is also undiscovered if RO0711401 might inhibit or accelerate its own metabolism with time. However, it was discovered that cortical levels of both drugs decreased across the days, while thalamic levels were constant across chronic treatment (8 days). The diminished levels in the cortex could indicate a contribution of pharmacokinetic tolerance to the loss of effects of RO0711401. Both PAMs were able to increase the expression of the respective subtypes in the thalamus and cortex on different days of drug treatment. Interestingly, RO0711401 in the thalamus on day eight of treatment increased the expression of mGlu1 receptors and mGlu5 receptors as well. In the cortex the drug increased mGlu1 receptor levels on day three. Contrastingly, treatment with VU0360172 increased the mGlu5 receptor expression in the thalamus, without any increase or decrease of mGlu1 receptor levels in thalamus and/or cortex. It may be that the amount of mGlu1-mGlu1 homodimers and mGlu1-mGlu5 heterodimers is predominant over mGlu5-mGlu5 homodimers in the cortex and in the thalamus of WAG/Rij. This would explain why the mGlu1 receptor PAM, RO0711401, caused changes in both mGlu1 and mGlu5 receptors, while on the contrary the mGlu5 receptor PAM, VU0360172, caused changes in mGluR5 only. Thereby, it might be that, VU0360172 did not develop pharmacodynamic tolerance due to it did not causes any changes in the mGlu1 receptor.

## Glutamatergic drugs in the cortico-thalamo-cortical network

Although the findings in Chapters 3 and 4 clearly suggest that VU0360172 might be develop for the treatment of absence epilepsy, it is still not known where within the C-T-C network activation of both mGlu1 or mGlu5 receptors reduces SWDs. In Chapter 5, this question is addressed by locally injecting either RO0711401 or VU0360172 in the cortex or in the thalamus. It was found that the effect in terms of SWD reduction might be caused by both intracortical and intrathalamic administration of mGlu1 or mGlu5 receptor PAMs. However, there was a different outcome between the thalamic and the cortical injection to RO0711401 and VU0360172. The two drugs were identically efficient in reducing SWDs into the cortex; conversely, the mGlu5 PAM, VU0360172, displayed a larger efficacy than the mGlu1 PAM, RO0711401, when administered in the thalamus.

*Cortex:* The identical effects of intracortically injected VU0360172 and RO0711401 can be explained by that in the somatosensory cortex, the two subtypes of group I mGlu receptors, localized postsynaptically on GABAergic interneurons, and have a familiar target. mGlu1 receptors are expressed by somatostatin-positive, calretinin-positive, and

calbindin-positive and mGlu5 receptors are discovered in both parvalbumin-positive GABAergic interneurons and somatostatin-positive (Stinehelfer et al., 2000; Sun et al., 2009). Therefore, it is possible that, a familiar target for the two PAMs could be a cell type expressing both mGlu1 and mGlu5 receptors, i.e. the somatostatin sensitive GABA-ergic interneurons. It may also be that pharmacological enhancement of mGluR1 or mGluR5 expressed by somatostatin sensitive and/or different types of regular spiking GABAergic interneurons decreases SWDs by increasing GABAergic inhibition toward pyramidal neurons.

*Thalamus:* VU0360172 showed a greater efficacy than RO0711401. Activation of either mGlu1 or mGlu5 receptors present in the VB and other thalamic neurons may restrain the occurrence of SWDs by negatively regulating the activity of T-type $Ca^{2+}$ channels on thalamo-cortical cells (Hildebrand et al., 2007; 2009).

However, a significant difference between mGlu1 and mGlu5 receptors is that only mGluR5 are located on astrocytes in the thalamus (Liu et al., 1998; Muly et al., 2003). Astrocytes are essential in the control of thalamic oscillations because they clear up extracellular glutamate and GABA from the extracellular space. In this way, they limit the activity of extra-synaptic glutamate and GABA receptors. The reduction in SWD seen with VU0360172 is in line with the pro-absence action observed after systemic administration of drugs that increase GABAergic neurotransmission, for instance tiagabine, which enhances the inhibitory GABAergic transmission at the synapses between RTN and VB neurons (Blumenfeld, 2005).

## GABAergic drug in cortico-thalamo-cortical network

Beyond glutamate, also GABA plays a key role in the control of SWDs. Indeed, local injections in cortex and thalamus with GABAergic drugs, and in combination with group I PAMs were performed (Chapter 5). This allowed us to establish the sites of action where GABA in its interaction with group I PAMs exerts its action. Rats were injected in cortex and thalamus with tiagabine. Tiagabine is known to enhance tonic-inhibition of thalamic relay neurons by inhibiting GABA re-uptake via the high affinity GABA transporter GAT-1, and aggravates the occurrence of SWDs after systemic injection therefore facilitating the endogenous activation of extra-synaptic GABA receptors (Coenen et al., 1995; Pow et al., 2005 Cope et al., 2009; Belelli et al., 2003; Errington et al., 2011).

## Tiagabine: which part of the C-T-C network is responsible for the increase/reduction of SWDs?

*Cortex:* tiagabine micro-infused in the cortex dose dependently decreased SWDs. It is thought by blocking the reuptake of GABA, thus enhancing GABAergic inhibition. The same result (decrease of SWDs) was obtained after the administration of either mGlu1 or mGlu5 PAM. This suggests that activation of both mGlu1 and mGlu5 receptors might suppress SWDs by increasing GABA-ergic inhibition onto pyramidal neurons, perhaps by stimulating somatostatin sensitive GABA-ergic neurons.

*Thalamus:* tiagabine micro-infused in the thalamus increased SWDs. As described in Chapters 1 and 5, drugs that increase inhibitory GABAergic transmission in WAG/Rij rats, such as tiagabine and vigabatrin, have a pro-absence effect. Therefore, the heightened incidence of SWDs after intra-thalamic injection of tiagabine was anticipated, and suggests that the pro-absence action in the thalamus prevails over the anti-absence activity in the cortex after systemic administration of tiagabine. It is proposed that thalamic administered tiagabine facilitated SWD by increasing tonic-inhibition of thalamic relay neurons by inhibiting GABA re-uptake via the high affinity GABA transporter GAT-1.

## Glutamatergic drugs plus GABAergic drugs in the cortico-thalamo-cortical network

*Cortex:* the combination of VU0360172 plus tiagabine in the cortex showed a reduction in SWDs. There was however, a small difference in terms of the duration of the SWD reduction between VU0360172 and tiagabine when compared to VU0360172 alone. The combination was still effective in reducing the incidence of SWDs till 40 min post-injection, while VU0360172 alone lasted 30 min post-injection (Figure 5A).Therefore, it was proposed by us that the reduced SWDs seen in both cases occurs by enhancing GABA-ergic inhibition onto pyramidal neurons (Figure 6A) and that the combination showed a small albeit significant prolongation of SWD suppressing effects.

*Thalamus:* the combination of VU0360172 plus tiagabine in the thalamus produced intriguing results. After co-administration, initially the SWD reducing effects of VU0360172 prevailed since the incidence of SWDs in the early post-injection time (10 min) was

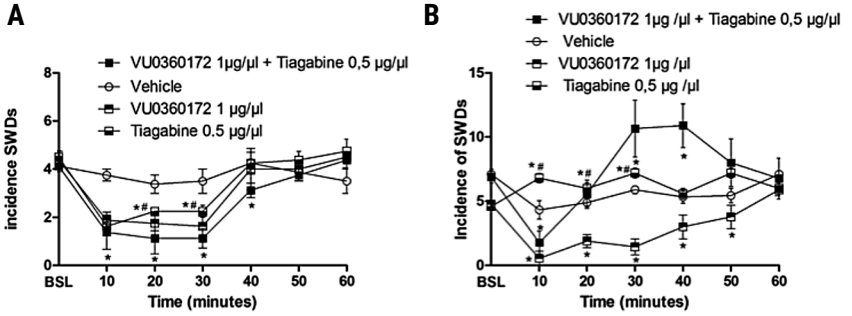



**Figure 5A-B** Effects of VU0360172 alone, tiagabine alone, the combination of VU0360172 and tiagabine and vehicle. infused in the thalamus and in the cortex (D'Amore et al., 2015). Notice the decrease in SWD incidence after both single drugs and the slightly prolonged effects of the combination in the cortex, while in the thalamus VU0360172 alone suppressed SWDs, tiagabine showed no effects, while the combitation showed that the action of VU0360172 was completely reversed in combination with tiagabine (D'Amore et al., 2015).

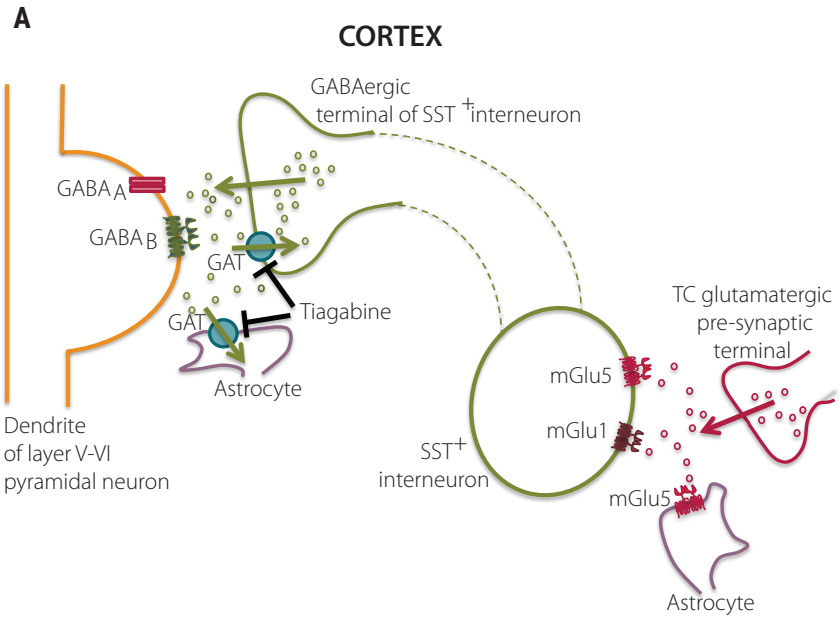



**Figure 6A-B** Mechanistic hypothesis of the role played by cortical (A) or thalamic (B) mGlu1 and mGlu5 receptors in the modulation of absence seizures. The diffusion of either GABA (rounded dots and arrows in green) or glutamate (rounded dots and arrows in red) within and out of the synaptic terminals and glia cells are depicted. In the S1po cortex, pharmacologic enhancement of mGlu1 or mGlu5 receptors might reduce absence seizures by activating somatostatin (SST)–positive GABAergic interneurons, and tiagabine might produce the same effect by enhancing GABA levels at the synapses between SST+ interneurons and pyramidal neurons. In the ventrobasal thalamus, tiagabine enhances absence seizures by increasing synaptic and extrasynaptic GABA levels, thereby facilitating the activity of T-type voltage-sensitive $Ca^{2+}$ channels. mGlu1 and mGlu5 receptors might protect against absence seizures by directing modulating T-type $Ca^{2+}$ channels or through a blockade of the glial glutamate transporter. Tiagabine might act via blockade of the presynaptic and glial GABA transporter. Data on the interaction between VU0360172 and tiagabine raise the possibility that mGlu5 receptors regulate the expression or activity of GAT-1 in GABAergic terminals and/or astrocytes (D'Amore et al., 2015).

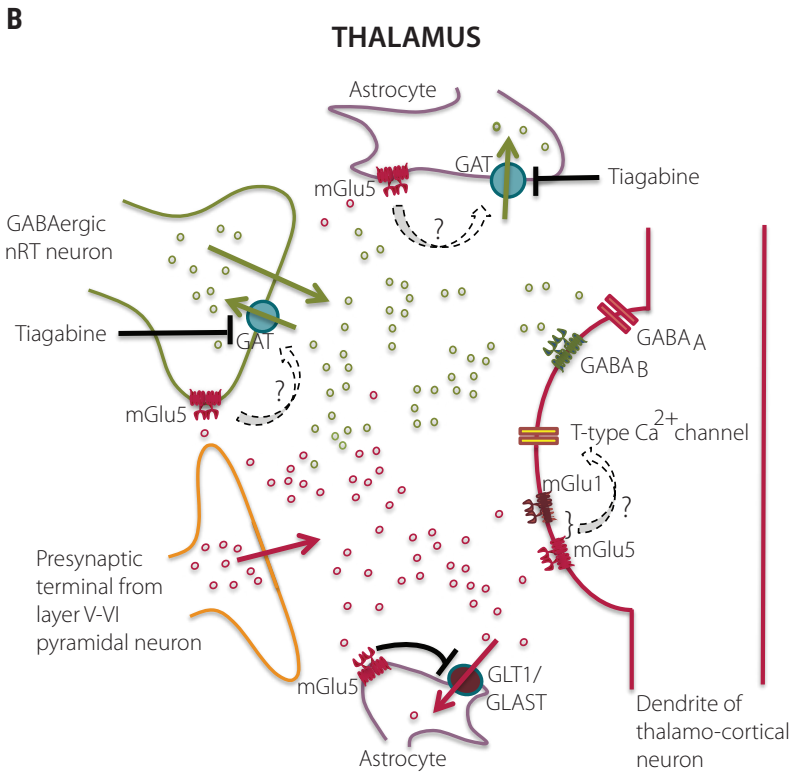



**Figure 6** Continued.

reduced. Next, this effect was lost at 20 min, and a pro-absence effect at 30 and 40 min occurred (see Figure 5 B). These results suggest that the activity of mGlu5 receptors is modulated by GABAergic transmission. It is known that the mGluR5 may control the activity of GAT-1 (Aronica et al., 2003) in astrocytes or in GABAergic fibers afferent to the ventrobasal thalamus. This implies that, in the presence of the GAT-1 inhibitor tiagabine activation of mGluR5 will not stimulate GABA reuptake, and the continuous enhancement in extracellular GABA will stop the anti-absence effect mediated by mGlu5-receptor stimulation (Figure 6B). In order to verify this hypothesis, experiments on tonic inhibition in thalamic slices or studies on GABA uptake alone and in the presence of mGlu5 PAM's in cultured of astrocytes in epileptic and non-epileptic rats are needed.

**Table 7** Action of mGlu and GABAergic drugs and their combination in the cortico-thalamo-cortical circuitry.

| | CORTEX | THALAMUS |
|---|---|---|
| VU0360172 | ↓ | ↓ |
| RO0711401 | ↓ | ↓ |
| tiagabine | ↓ | ↑ |
| VU0360172 + tiagabine | ↓ | ↓ ↑ |
| ETX | ↓ | ↓ |

## VU0360172 vs. ethosuximide in the cortico-thalamo-cortical network

As described in Chapter 1, Chapter 6, and Chapter 7, ETX is a first choice antiabsence medicine (Leresche et al., 1998). However when infused into either the VB or RTN of GAERS (Marescaux et al., 1992; Danober et al., 1998) it had only a moderate and delayed decrease in SWDs, when compared to the instantaneous and labeled decrease observed after systemic injection (Richards et al., 2003). When injected into the somatosensory cortex it did result in a direct and almost full interruption of SWDs, equivalent to that found after systemic or intracerebroventricular administration (Marescaux et al., 1984; van Luijtelaar et al., 2013; Richards et al., 2003; Gurbanova et al., 2006). In Chapter 5, it has been shown that VU0360172 reduced SWDs equally well when locally injected in cortex and thalamus. Taking these data together, it may be possible to highlight a difference in terms of SWD reduction between ETX and VU0360172. VU0360172 targets both locations equally effective, and this is less the case for ETX. Therefore it could be proposed that this factor may contribute to successful seizure suppression of mGluR group I PAMs (see Table 7).

## Need for new treatment options in absence epilepsy

In Chapter 6, "Is there a future for mGlu5 PAMs in absence epilepsy? A comparison between VU0360172 and Ethosuximide", emphasizes the need for new treatment options in absence epilepsy. ETX is the drug of choice in the treatment of various types of absence seizures. However, there is room for other anti-absence drugs considering that not all subjects become seizure free and in about 47% of subjects, ETX therapy failed (Tenney

and Glauser, 2013). Another issue is that since it is frequently hard to get good seizure control with only a single antiepileptic drug, interaction studies among several antiepileptic drugs are pointed out. The review highlighted that ETX had infra-additive effects with valproic acid in WAG/Rij rats, so combination studies of PAMs with ETX and valproic acid are indicated. A newly discovered action of chronic ETX, but also with some other compounds, is antiepileptogenesis in the genetic absence models. Therefore it is also important to investigate whether antiepileptogenesis is also achievable with group I PAMs and whether antiepileptogenesis affects the sensitivity of the other anti-absence drugs. Thereby, this chapter emphasized that a good candidate to investigate antiepileptogenesis in combination with ETX might be the mGlu5 PAM, VU0360172.

## Could VU0360172 be combined with other AED?

In Chapter 7, the anti-absence and anti-epileptogenetic effects of ETX and the interaction between VU0360172 and ETX was evaluated. In this case ETX was chronically administered for 4 months in order to induce anti-epileptogenesis, next it was evaluated whether and how VU0360172 interacted with ETX. The anti-absence and antiepileptogenic effects of ETX were successfully established, in line with outcomes of earlier studies (Blumenfeld et al., 2008; Sarkisova et al., 2010; Russo et al., 2010, 2011; van Luijtelaar et al., 2013). VU0360172, as expected (Chapters 3, 4 and 5), reduced SWDs in a dose and time dependent way in the untreated control group. However, the same injections with VU0360172 in chronically ETX treated rats induced a small reduction in SWD incidence throughout the whole recording session without returning to the baseline. This effect was found both during and after ETX treatment had stopped. It seems that the combination of chronic ETX plus acute VU0360172 seemed to have larger effects than ETX alone and therefore a better pharmacological profile in terms of SWD diminishment when compared to ETX alone, although the size of the SWD reducing effects of VU0360172 was diminished compared to the size of the effects in the water treated control group. The favourable profile is important if we take into consideration the life-compliance of the patients with respect to the dose/drug administration protocol. Considering the decrease in the sensitivity of VU0360172 in the chronic treated rats, western blots was done measuring the protein for mGluR5 in this group. The results did not show a down- or up-regulation of mGlu5 receptor for the chronic treated ETX group when compared with the age matched non-treated control group. This suggests that this part of the system is not modified and indeed VU0360172 kept some of its anti-absence effects.

## Hypothetical signaling pathway of ETX

Identification of primary cell signaling pathways that trigger different downstream mechanisms mediating epileptogenesis are beginning to gain important roles, and many current research has been focused on this signaling identification (Dichter, 2006; Pitkänen and Lukasiuk, 2011). The rapamycin (mTOR) pathway is one signal transduction system

that has gained attention as a significant regulator of cellular changes concerned in epileptogenesis (McDaniel & Wong, 2011; Russo et al., 2012; 2013; Zeng et al., 2009a). Quite a few studies have showed a potential role of mTOR signaling in epilepsy and in particular, epileptogenesis in both convulsive and non-convulsive epilepsies. A recent study has shown that acute and sub-chronic administration of the mTOR inhibitor rapamycin decreased SWD activity. Moreover, in the same study it has been found that rapamycin has antiepileptogenic effects in WAG/Rij rats, most likely through mTOR inhibition as well (Russo et al., 2013). Moreover, it is well known that, mTOR affect a variety of cellular and molecular processes, such as ion channel expression and neurotransmitter receptor, and apoptosis, neurogenesis and synaptic plasticity, all of which are known to influence neuronal excitability and epileptogenesis (Tang et al., 2002; Kumar et al., 2005; Wang et al., 2006). However, the exact mechanisms involved in these processes have not yet been clarified (Galanopoulou et al., 2012; Russo et al., 2012; Wong, 2013). Interestingly, vigabatrin has the same kinds of antiepileptogenic action in WAG/Rij rats through inhibition of the mTOR pathway (Russo et al., 2011; Zhang et al., 2013).

Although, the engagement of the mTOR pathway in epileptogenic mechanisms has been discussed in detail (Cho, 2011), investigations to define the mechanisms by which rapamycin may influence both the initial development and the ongoing maintenance of epilepsy, and to determine the clinical utilization of rapamycin for preventing epilepsy still need to be addressed.

## Has ETX and VU0360172 mTOR a common signaling pathway?

A common target between ETX and VU0360172 may be the mTOR pathway. It is known that the mGluR5 antagonist, CHPG, activated the mTOR pathway (Cao et al., 2015). It is therefore possible to speculate that VU0360172 is able to inhibit the activity of the mTOR pathway. So it might be that VU0360172, although it maintained its anti-absence effect during and after ETX treatment (see Chapter 6 and above), lost its common dose-dependency effect due to the already inhibited mTOR signaling by chronic ETX treatment.

## ETX and VU0360172; behavior and motivation

Most AED such as ETX, VPA, and LTG have a negative impact on cognition (Conant et al., 2010; Pavone, 2001). Moreover, the antiepileptogenic effects of chronic ETX were accompanied by a decrease in depressive-like behavior in WAG/Rij rats (Sarkisova et al., 2010; van Luijtelaar et al., 2013). In Chapter 7, it was also investigated whether cognitive aspects have been modified as a consequence of chronic ETX treatment. Rats were exposed to a cue discrimination learning task in a Y-maze and in a sucrose motivation task during and after chronic treatment. The outcomes showed that chronic ETX enhanced motivation to collect sucrose pallets, and this was followed by an increase in cued discrimination learning. The cognitive enhancing effects of ETX, as found at the end of the experiment (when the rats had received also the challenge with VU0360172), might

be mediated to the antidepressant action of ETX as expressed by an increase in the rewarding properties of sucrose pallets. As for the mGluR5, the activation with a PAM (see Chapter 1) might increase at the same time both forms of synaptic plasticity (long-term potentiation and long-term depression of synaptic strength), thereby enhancing cognitive function (Noetzel et al., 2012; Ayala et al., 2009). This supplies an ideal profile for enhancing cognitive function, and indeed mGluR5 PAMs increase cognitive function in several rodent models (Chan et al., 2008; Gastambide et al., 2013). It is also known that comparable PAMS are proposed for the treatment of psychosis and cognitive disturbances in schizophrenic patients (Cleva et al., 2011).

## mGluR drugs in clinical use and future development

Several mGluR ligands are under clinical development for the treatment of different nervous disorders such as; Parkinson's disease, schizophrenia, Fragile-X syndrome, chronic pain, and generalized anxiety disorder. Those compounds, selectively target Group I or Group II mGluRs, principally mGluR2/3 and mGluR5 (Yasuhara et al., 2010; Nicoletti et al., 2011; Levenga et al., 2011; Fell et al., 2012). The mGluR5 antagonists MPEP and correlated mGluR5 NAMs have strong efficacy in plural animal models of anxiolytic activity (Swanson et al., 2005; Pecknold et al., 1982). Moreover, outcomes from animal studies recommend that, mGluR5 NAMs also have utility in the treatment of Fragile-X syndrome, because the primary mutation-giving origin to the sickness leads to enhanced mGluR5 function (Ronesi et al., 2008). But also, mGluR5 NAM might for instance normalize the excessive protein synthesis, which might give origin to symptoms correlated with Fragile X syndrome (Bear et al., 2004). MGluR5 NAM might also be used for chronic pain, depression and some neurodegenerative disorders (Yan et al., 2005; Slassi et al., 2005; Lehmann, 2008). Next to the efficacy of mGluR5 NAMs, mGluR5 PAMs might have also advantage in the treatment of schizophrenia (Conn et al., 2008; Moghaddam, 2004; Kinney et al., 2005; Liu et al., 2008; Darrah et al., 2008).

## Conclusions

Acute and chronic treatment with positive allosteric modulator of mGlu5 metabotropic glutamate receptors, VU0360172, decrease the SWD occurrence in the WAG/Rij rat model of absence epilepsy without development of tolerance and/or clear behaviour disturbances. RO0711401 was also effective acutely and in cortex and thalamus, but tolerance developed quickly. Therefore, VU0360172 might be preferentially developed in the near future for the treatment of absence epilepsy. Secondly, opposite effects of targeting group I mGluR were found in WAG/Rij rats compared to results targeting the same receptors with orthostatic agonists and antagonists obtained in another genetic absence model, the lethargic mice; many factors such as genetic background, behavioural phenotype, and differences in mGlu receptor distributions and densities in thalamus and cortex, might be the cause of these differences. Thirdly, as for the mechanisms, in cortex

VU0360172 might enhance GABAergic inhibition, conversely, in thalamus, the activity of mGlu5 receptors might reduce the enhanced tonic inhibition and the action of VU0360172 might be affected by the availability of extra-synaptic GABA. Fourthly, VU0360172 targets both locations equally effective (cortex and thalamus) while the drug of choice, ethosuximide, is less effective in the thalamus when compared to the injection in the cortex. Therefore, it is proposed that this contributes to the efficacy of mGlu5 PAM in successful seizure suppression. But also that VU0360172 might be combined with other anti-epileptic drugs such as ETX for a better seizure control.

## Direction for future research

Future research is needed in order to settle out whether or not the mGlu5 receptor PAMs, might be introduced for clinical use, and are safe in combination with other drugs used for the treatment of absence epilepsy.

In order to predict the safety and tolerability profile of VU0360172, neurotoxic studies need to be done with higher doses of the mGlu5 PAM, such as the measurement of stereotyped motor coordination tasks e.g. rotarod. Given the notion that the PAMs might modulate glutamate, but also that VU0360172 might act via enhancing GABA-ergic transmission, its sedative properties should be investigated.

If group I PAM receptor can be further developed as an anti-absence drug, the preclinical profile of VU0360172 in other generalized and focal epilepsy models, including mesial temporal epilepsy, needs to be determined. The fact that it may aggravate the action of GABA-ergic interneurons, as was established in the focal excitable area in the WAG/Rij model, could point toward this.

Further studies should be aimed at establishing whether VU0360172 has antiepileptogenic effects and pharmacological challenges after chronic VU0360172 treatment with ETX, and/or other AEDs should be performed to establish whether chronic treatment can modulate the direction of drug's effects.

Since it is frequently hard to achieve good seizure control with just a single antiepileptic drug, interaction studies among various antiepileptic drugs are suggested. Acute and chronic studies with VU0360172 and other AED must be evaluated.

Research towards mechanisms regarding antiepileptogenesis need to be done. As described in this thesis, the mTOR signaling pathway may be involved in antiepileptogenesis. Additionally, acute studies with VU0360172 and ETX could be helpful in clarifying the interaction seen between the two drugs and the hypothetical involvement of the mTOR pathway.

Future studies should be focused on how and in what ways VU0360172 interacts with GABA. It is proposed that studies towards GABA reuptake in cultured astrocytes or on tonic inhibition in thalamic slices in epileptic and non-epileptic rats under normal circumstances and or in the presence of VU0360172.

To review our knowledge on the desensitization of mGlu receptors, and perhaps discover the real nature of the tolerance developed to RO0711401, and not versus VU0360172. Studies are needed of the molecules involved in desensitization and internalization of mGlu1 and mGlu5 receptors, such as the G-protein coupled receptor kinases and β-arrestin.

# References


Alexander GM, Godwin DW. 2006. Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res. 71, 1-22.

Aronica E, Gorter JA, Ijlst-Keizers H, Rozemuller AJ, Yankaya B, Leenstra S, Troost D. 2003. Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. Eur J Neurosci. 17, 2106-18.

Ayala, J. E. Chen Y, Banko JL, Sheffler DJ, Williams R, Telk AN, Watson NL, Xiang Z, Zhang Y, Jones PJ, Lindsley CW, Olive MF, Conn PJ. 2009. MGluR5 positive allosteric modulators facilitate both hippocampal LTP and LTD and enhance spatial learning. Neuropsycopharmacology 34, 2057-2071.

Barton ME, Peters SC, Shannon HE. 2003. Comparison of the effect of glutamate receptor modulators in the 6 Hz and maximal electroshock seizure models. Epilepsy Res. 56, 17-26.

Bear MF, Huber KM, Warren ST. 2004. The mGluR theory of fragile X mental retardation. Trends Neurosci. 27, 370-7.

Belelli D, Herd MB. 2003. The contraceptive agent Provera enhances GABA(A) receptor-mediated inhibitory neurotransmission in the rat hippocampus: evidence for endogenous neurosteroids? J Neurosci. 23, 10013-20.

Blumenfeld H. 2005. Cellular and network mechanisms of spike-wave seizures. Epilepsia 46, 21-33.

Blumenfeld H, Klein JP, Schridde U, Vestal M, Rice T, Khera DS, Bashyal C, Giblin K, Paul-Laughinghouse C, Wang F, Phadke A, Mission J, Agarwal RK, Englot DJ, Motelow J, Nersesyan H, Waxman SG, Levin AR. 2008. Early treatment suppresses the development of spike-wave epilepsy in a rat model. Epilepsia 49, 400-9.

Bruno V, Battaglia G, Copani A, D'Onofrio M, Di Iorio P, De Blasi A, Melchiorri D, Flor PJ, Nicoletti F. 2001. Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J Cereb Blood Flow Metab. 21, 1013-33.

Burgess DL, Noebels JL. 1999. Single gene defects in mice: the role of voltage-dependent calcium channels in absence models. Epilepsy Res. 36, 111-22.

Cao L, Tian Y, Jiang Y, Zhang GJ, Lei H, Di ZL. 2015. Down-regulation of Homer1b/c protects against chemically induced seizures through inhibition of Mtor signaling. Cell Physiol Biochem. 35, 1633-42.

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJ, Bymaster FP, Felder CC. 2008. Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc. Natl Acad. SCI. USA 105, 10978-83.

Chapman AG, Nanan K, Williams M, Meldrum BS. 2000. Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893). Neuropharmacol. 39, 1567-74.

Chapman AG, Yip PK, Yap JS, Quinn LP, Tang E, Harris JR, Meldrum BS. 1999. Anticonvulsant actions of LY 367385 ((+)-2-methyl-4-carboxyphenylglycine) and AIDA ((RS)-1-aminoindan-1,5-dicarboxylic acid). Eur J Pharmacol. 368, 17-24.

Cheong E, Zheng Y, Lee K, Lee J, Kim S, Sanati M, Lee S, Kim YS, Shin HS. 2009. Deletion of phospholipase C beta4 in thalamocortical relay nucleus leads to absence seizures. Proc Natl Acad Sci U S A. 106, 21912-7.

Cho CH. 2011. Frontier of epilepsy research - mTOR signaling pathway. Exp Mol Med. 43, 231-74.

Cleva RM, Olive MF. 2011. Positive allosteric modulators of type 5 metabotropic glutamate receptors (mGluR5) and their therapeutic potential for the treatment of CNS disorders. Molecules 16, 2097-106.

Coenen AM, Blezer EH, van Luijtelaar EL. 1995. Effects of the GABA-uptake inhibitor tiagabine on electroencephalogram, spike-wave discharges and behaviour of rats. Epilepsy Res. 21, 89-94.

Coenen AM, van Luijtelaar EL. 2003. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav Genet. 33, 635-55.

Conant L, Wilfong A, Inglese C, Schwarte A. 2010. Dysfunction of executive and related processes in childhood absence epilepsy. Epilepsy Behav. 18, 414-23.

Conn PJ, Lindsley CW, Jones C. 2008. Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. Trends Pharmacol. Sci. 30, 25–31.

Cope DW, Di Giovanni G, Fyson SJ, Orbán G, Errington AC, Lorincz ML, Gould TM, Carter DA, Crunelli V. 2009. Enhanced tonic GABA inhibition in typical absence epilepsy. Nat Med. 15, 1392-8.

Danober L, Deransart C, Depaulis A, Vergnes M, Marescaux C. 1998. Pathophysiological mechanisms of genetic absence epilepsy in the rat. Prog Neurobiol. 55, 27-57.

Darrah JM, Stefani MR, Moghaddam B. 2008. Interaction of N-methyl-D-aspartate and group 5 metabotropic glutamate receptors on behavioral flexibility using a novel operant set-shift paradigm. Behav. Pharmacol. 19, 225-34.

Dichter MA. 2006. Models of epileptogenesis in adult animals available for antiepileptogenesis drug screening. Epilepsy Res. 68, 31-5.

Errington AC, Di Giovanni G, Crunelli V, Cope DW. 2011. mGluR control of interneuron output regulates feedforward tonic GABAA inhibition in the visual thalamus. J Neurosci. 31, 8669-80.

Fell MJ, McKinzie DL, Monn JA, Svensson KA. 2012. Group II metabotropic glutamate receptor agonists and positive allosteric modulators as novel treatments for schizophrenia. Neuropharmacology 62, 1473-83.

Galanopoulou AS, Gorter JA, Cepeda C. 2012. Finding a better drug for epilepsy: the mTOR pathway as an antiepileptogenic target. Epilepsia 53, 1119-30.

Gastambide F, Gilmour G, Robbins TW, Tricklebank MD. 2013.The mGlu5 positive allosteric modulator LSN2463359 differentially modulates motor, instrumental and cognitive effects of NMDA receptor antagonists in the rat. Neuropharmacology 64, 240-247.

Gurbanova AA, Aker R, Berkman K, Onat FY, van Rijn CM, van Luijtelaar G. 2006. Effect of systemic and intracortical administration of phenytoin in two genetic models of absence epilepsy. Br J Pharmacol. 148, 1076-82.

Hildebrand ME, David LS, Hamid J, Mulatz K, Garcia E, Zamponi GW, Snutch TP. 2007. Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11-coupled muscarinic acetylcholine receptors. J Biol Chem. 282, 21043-55.

Hildebrand ME, Isope P, Miyazaki T, Nakaya T, Garcia E, Feltz A, Schneider T, Hescheler J, Kano M, Sakimura K, Watanabe M, Dieudonn S, Snutch TP. 2009. Functional coupling between mGluR1 and Cav3.1 T-type calcium channels contributes to parallel fiber-induced fast calcium signaling within Purkinje cell dendritic spines. J Neurosci 29, 9668-82.

Hosford DA, Lin FH, Kraemer DL, Cao Z, Wang Y, Wilson JT Jr. 1995. Neural network of structures in which GABAB receptors regulate absence seizures in the lethargic (lh/lh) mouse model. J Neurosci. 15, 7367-76.

Kinney GG, O'Brien JA, Lemaire W, Burno M, Bickel DJ, Clements MK, Chen TB, Wisnoski DD, Lindsley CW, Tiller PR, Smith S, Jacobson MA, Sur C, Duggan ME, Pettibone DJ, Conn PJ, Williams DL Jr. 2005. A novel selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 has in vivo activity and antipsychotic-like effects in rat behavioral models. J. Pharmacol. Exp. Ther. 313, 199-206.

Kumar V, Zhang MX, Swank MW, Kunz J, Wu GY. 2005. Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. J Neurosci. 25, 11288-99.

Lehmann A. 2008. Novel treatments of GERD: focus on the lower esophageal sphincter. Eur. Rev. Med. Pharmacol. Sci. 12,103-10.

Leresche N, Parri HR, Erdemli G, Guyon A, Turner JP, Williams SR, Asprodini E,Crunelli V. 1998. On the action of the anti-absence drug ethosuximide in the rat and cat thalamus. J Neurosci. 18, 4842-53.

Levenga J, Hayashi S, de Vrij FM, Koekkoek SK, van der Linde HC, Nieuwenhuizen I, Song C, Buijsen RA, Pop AS, Gomezmancilla B, Nelson DL, Willemsen R, Gasparini F, Oostra BA. 2011. AFQ056, a new mGluR5 antagonist for treatment of fragile X syndrome. Neurobiol Dis. 42, 311-7.

Liu F, Grauer S, Kelley C, Navarra R, Graf R, Zhang G, Atkinson PJ, Popiolek M, Wantuch C, Khawaja X, Smith D, Olsen M, Kouranova E, Lai M, Pruthi F, Pulicicchio C, Day M, Gilbert A, Pausch MH, Brandon NJ, Beyer CE, Comery TA, Logue S, Rosenzweig-Lipson S, Marquis KL. 2008. ADX47273 [S-(4-fluoro-phenyl)-{3-[3- (4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piper idin-1-yl}-methanone]: a novel metabotropic glutamate receptor 5-selective positive allosteric modulator with preclinical antipsychotic-like and precognitive activities. J. Pharmacol. Exp. Ther. 327, 827-39.

Liu L, Zheng T, Morris MJ, Wallengren C, Clarke AL, Reid CA, Petrou S, O'Brien TJ. 2006. The mechanism of carbamazepine aggravation of absence seizures. J Pharmacol Exp Ther. 319, 790-8.

Liu XB, MuÇoz A, Jones EG. 1998. Changes in subcellular localization of metabotropic glutamate receptor subtypes during postnatal development of mouse thalamus. J Comp Neurol. 395, 450-65.

Lüttjohann A, van Luijtelaar G. 2015. Dynamics of networks during absence seizure's on- and offset in rodents and man. Front Physiol. 5, 6:16.

Lüttjohann A, van Luijtelaar G. 2012. The dynamics of cortico-thalamo-cortical interactions at the transition from pre-ictal to ictal LFPs in absence epilepsy. Neurobiol Dis. 47, 49-60.

Marescaux C, Micheletti G, Vergnes M, Depaulis A, Rumbach L, Warter JM. 1984. A model of chronic spontaneous petit mal-like seizures in the rat: comparison with pentylenetetrazol-induced seizures. Epilepsia 25, 326-31.

Marescaux C, Vergnes M, Bernasconi R. 1992. GABAB receptor antagonists: potential new anti-absence drugs. J Neural Transm Suppl. 35,179-88.

McDaniel SS, Wong M. 2011. Therapeutic role of mammalian target of rapamycin (mTOR)inhibition in preventing epileptogenesis. Neurosci Lett. 497,231-9.

Meeren HK, Pijn JP, Van Luijtelaar EL, Coenen AM, Lopes da Silva FH. 2002. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J Neurosci. 22,1480-95.

Miyata M, Kashiwadani H, Fukaya M, Hayashi T, Wu D, Suzuki T, Watanabe M, Kawakami Y. 2003. Role of thalamic phospholipase C[beta]4 mediated by metabotropic glutamate receptor type 1 in inflammatory pain. J Neurosci. 23, 8098-108.

Moghaddam B. 2004. Targeting metabotropic glutamate receptors for treatment of the cognitive symptoms of schizophrenia. Psychopharmacology (Berl.) 174, 39–44.

Muly EC, Maddox M, Smith Y. 2003. Distribution of mGluR1alpha and mGluR5 immunolabeling in primate prefrontal cortex. J Comp Neurol. 467, 521-35.

Ngomba RT, Ferraguti F, Badura A, Citraro R, Santolini I, Battaglia G, Bruno V, De Sarro G, Simonyi A, van Luijtelaar G, Nicoletti F. 2008. Positive allosteric modulation of metabotropic glutamate 4 (mGlu4) receptors enhances spontaneous and evoked absence seizures. Neuropharmacology. 54, 344-54.

Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP. 2011. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60, 1017-41.

Noetzel, M.J, Rook JM, Vinson PN, Cho HP, Days E, Zhou Y, Rodriguez AL, Lavreysen H, Stauffer SR, Niswender CM, Xiang Z, Daniels JS, Jones CK, Lindsley CW, Weaver CD, Conn PJ. 2012. Functional impact of allosteric agonist activity of selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 in regulating central nervous system function. Mol. Pharmacol. 81, 120-133.

O'Brien JA, Lemaire W, Wittmann M, Jacobson MA, Ha SN, Wisnoski DD, Lindsley CW, Schaffhauser HJ, Rowe B, Sur C, Duggan ME, Pettibone DJ, Conn PJ, Williams DL Jr. 2004. A novel selective allosteric modulator potentiates the activity of native metabotropic glutamate receptor subtype 5 in rat forebrain. J. Pharmacol. Exp. Ther. 309, 568-77.

Pavone P, Bianchini R, Trifiletti RR, Incorpora G, Pavone A. 2001. Parano E. Neuropsychological assessment in children with absence epilepsy. Neurology 56, 1047-51.

Pecknold JC, McClure DJ, Appeltauer L, Wrzesinski L, Allan T. 1982. Treatment of anxiety using fenobam (a nonbenzodiazepine) in a double-blind standard (diazepam) placebo-controlled study. J. Clin. Psychopharmacol. 2, 129-33.

Pitkänen A, Lukasiuk K. 2011. Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol. 10, 173-86.

Polack PO, Mahon S, Chavez M, Charpier S. 2009. Inactivation of the somatosensory cortex prevents paroxysmal oscillations in cortical and related thalamic neurons in a genetic model of absence epilepsy. Cereb Cortex 19, 2078-91.

Pow DV, Sullivan RK, Williams SM, Scott HL, Dodd PR, Finkelstein D. 2005. Differential expression of the GABA transporters GAT-1 and GAT-3 in brains of rats, cats, monkeys and humans. Cell Tissue Res. 320, 379-92.

Reiter E, Lefkowitz RJ. 2006. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. Trends Endocrinol Metab. 17, 159-65.

Richards DA, Manning JP, Barnes D, Rombola L, Bowery NG, Caccia S, Leresche N, Crunelli V. 2003. Targeting thalamic nuclei is not sufficient for the full anti-absence action of ethosuximide in a rat model of absence epilepsy. Epilepsy Res. 54, 97-107.

Rodriguez AL, Grier MD, Jones CK, Herman EJ, Kane AS, Smith RL, Williams R, Zhou Y, Marlo JE, Days EL, Blatt TN, Jadhav S, Menon UN, Vinson PN, Rook JM, Stauffer SR, Niswender CM, Lindsley CW, Weaver CD, Conn PJ. 2010. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. Mol Pharmacol. 78, 1105-23.

Ronesi JA, Huber KM. 2008. Homer interactions are necessary for metabotropic glutamate receptorinduced long-term depression and translational activation. J. Neurosci. 28, 543-47.

Russo E, Citraro R, Constanti A, De Sarro G. 2012. The mTOR signaling pathway in the brain: focus on epilepsy and epileptogenesis. Mol Neurobiol. 46, 662-81.

Russo E, Citraro R, Donato G, Camastra C, Iuliano R, Cuzzocrea S, Constanti A, De Sarro G. 2013. mTOR inhibition modulates epileptogenesis, seizures and depressive behavior in a genetic rat model of absence epilepsy. Neuropharmacology 69, 25-36.

Russo E, Citraro R, Scicchitano F, De Fazio S, Di Paola ED, Constanti A, De Sarro G. 2010. Comparison of the antiepileptogenic effects of an early long-termtreatment with ethosuximide or levetiracetam in a genetic animal model of absenceepilepsy. Epilepsia 51, 1560-9.

Russo E, Citraro R, Scicchitano F, Urzino A, Marra R, Rispoli V, De Sarro G. 2011. Vigabatrin has antiepileptogenic and antidepressant effects in an animal model of epilepsy and depression comorbidity. Behav Brain Res. 225, 373-6.

Shannon HE, Peters SC, Kingston AE. 2005. Anticonvulsant effects of LY456236, a selective mGlu1 receptor antagonist. Neuropharmacology 1, 188-95.

Sarkisova KY, Kuznetsova GD, Kulikov MA, van Luijtelaar G. 2010. Spike-wave discharges are necessary for the expression of behavioral depression-likesymptoms. Epilepsia 51, 146-60.

Slassi A, Isaac M, Edwards L, Minidis A, Wensbo D, Mattsson J, Nilsson K, Raboisson P, McLeod D, Stormann TM, Hammerland LG, Johnson E. 2005. Recent advances in non-competitive mGlu5 receptor antagonists and their potential therapeutic applications. Curr. Top. Med. Chem. 5, 897-911.

Stinehelfer S, Vruwink M, Burette A. 2000. Immunolocalization of mGluR1alpha in specific populations of local circuit neurons in the cerebral cortex. Brain Res. 861, 37-44.

Sun QQ, Zhang Z, Jiao Y, Zhang C, Szab G, Erdelyi F. 2009. Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. J Neurophysiol. 101, 2679-92.

Swanson CJ, Bures M, Johnson MP, Linden A-M,Monn JA, SchoeppDD. 2005. Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. Nat. Rev. Drug Discov. 4, 131–44.

Tang SJ, Reis G, Kang H, Gingras AC, Sonenberg N, Schuman EM. 2002. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc Natl Acad Sci U S A 99, 467-72.

Tenney JR, Glauser TA. 2013. The current state of absence epilepsy: can we have your attention? Epilepsy Curr. 13, 135-40.

van Luijtelaar G, Mishra AM, Edelbroek P, Coman D, Frankenmolen N, Schaapsmeerders P. P, Covolato G, Danielson N, Niermann H, Janeczko K, Kiemeneij A, Burinova J, Bashyal C, Coquillette M, Lüttjohann A, Hyder F, Blumenfeld H, van Rijn CM. 2013. Anti-epileptogenesis: Electrophysiology, diffusion tensor imaging and behavior in a genetic absence model. Neurobiol Dis. 60, 126-138.

Vieira E, Huwyler J, Jolidon S, Knoflach F, Mutel V, Wichmann J. 2009. Fluorinated 9H-xanthene-9-carboxylic acid oxazol-2-yl-amides as potent, orally available mGlu1 receptor enhancers. Bioorg Med Chem Lett. 19, 1666-9.

Wang X, Sun DF, Fang JY. 2006. Research advances on the relationship of PI3-kinase/Akt/mTOR pathway and epigenetic modification. Yi Chuan 28, 1585-90.

Watanabe M, Nakamura M, Sato K, Kano M, Simon MI, Inoue Y. 1998. Patterns of expression for the mRNA corresponding to the four isoforms of phospholipase Cbeta in mouse brain. Eur J Neurosci. 10, 2016-25.

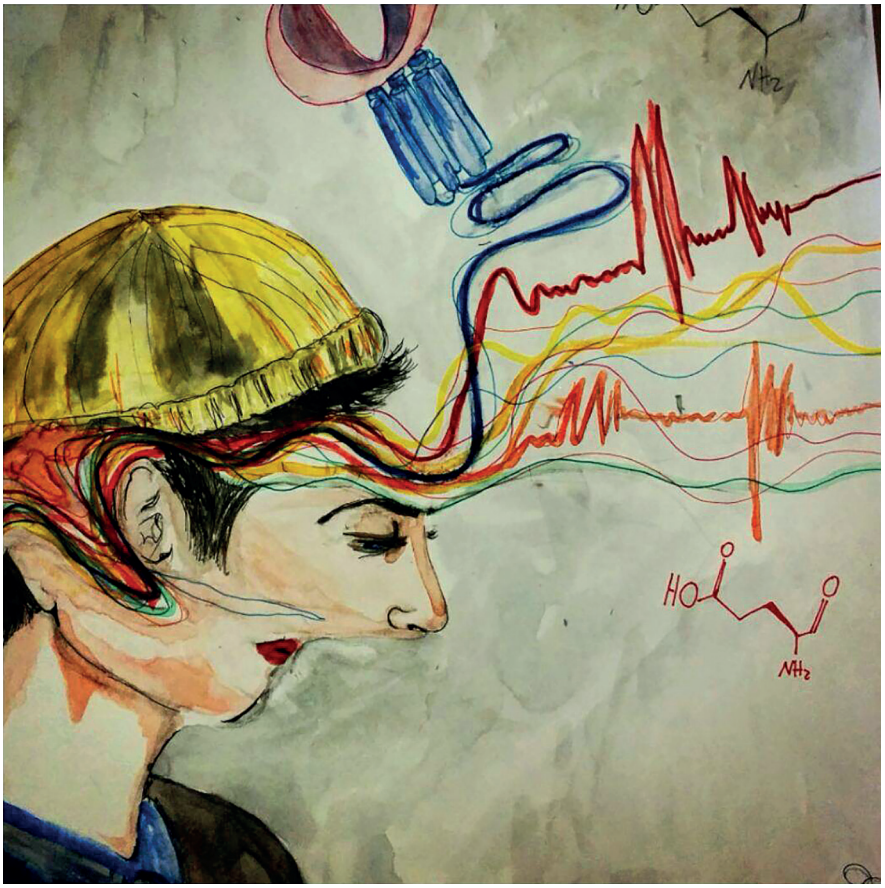
Wong M. 2013. Mammalian target of rapamycin (mTOR) pathways in neurological diseases. Biomed J. 36, 40-50.

Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP. 2005. Suppression of two major fragile X syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. Neuropharmacology 49, 1053–66.

Yasuhara A, Chaki S. 2010. Metabotropic glutamate receptors: potential drug targets for psychiatric disorders. Open Med. Chem. J 4, 20-36.

Zeng LH, Rensing NR, Wong M. 2009. The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. J Neurosci. 29, 6964-72.

Zhang B, McDaniel SS, Rensing NR, Wong M. 2013. Vigabatrin inhibits seizures and mTOR pathway activation in a mouse model of tuberous sclerosis complex. PLoS One 8, e57445.




# Addendum

# English Summary

Childhood absence epilepsy (CAE) accounts for 2% to 8% of patients with epilepsy, with the main cause being predominantly genetic. About one-third of the families of children with CAE report a family history of similar seizures. The siblings of children with CAE have about a 10% chance of developing epilepsy. CAE is considered to be one of the relatively benign childhood epilepsies. It typically begins between 4 and 8 years of age and is characterized by daily, frequent, brief staring spells, often with the eyes fluttering and the child being unresponsive to external stimuli. New anti-epileptic drugs are badly needed considering that not all subjects (57%–74%) become seizure-free, and in about 47% of subjects Ethosuximide (ETX) therapy, one of the preferred anti-absence drugs, failed. New anti-absence drugs might target glutamatergic and/or GABA-ergic neurotransmission, the key players in the network involved in the cortico-thalamo-cortical (C-T-C) oscillations responsible for the highly stereotyped spike-wave discharges (SWDs). There is quite some evidence that in rodent models the facial area of the somatosensory cortex contains a hyper-excitable area from which the SWDs originate. The presence of metabotropic glutamate receptors (mGluRs) within the C-T-C circuit suggests that these receptors are possible targets for the treatment of absence epilepsy. Moreover, since targeting mGluRs might be less toxic than targeting ionotropic glutamate receptors, it was imperative to explore the role of mGluRs in absence epilepsy, also considering that drugs targeting mGluR have been shown to exert pro- or anti-epileptic effects in various seizure and epilepsy models. A final reason was also that allosteric modulators of group I have been shown to be safe and have already entered clinical development for other neurological and psychiatric disorders.

In particular, the roles of group I mGlu receptors—both mGlu1 and mGlu5 belong to this category—will be investigated. They are preferentially expressed in the peripheral portion of the postsynaptic density, where they cause excitatory responses and control mechanisms of synaptic plasticity.

The experiments, as described in this thesis, investigated the involvement of group I mGluR in the pathophysiology of absence epilepsy, and this was done in a well-validated and frequently used model: rats of the WAG/Rij strain. The role of mGluR1 and mGluR5 was investigated by establishing their expression and signaling in the brain regions where the pathological SWDs initiate, spread and are maintained: the C-T-C circuitry. Most often, symptomatic rats were compared with non-symptomatic rats and age-matched control (ACI) rats. Furthermore, pharmacological EEG-behavioral studies were carried out with the aim to establish whether positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs) of this group can be developed as putative anti-absence drugs.

First, in **Chapter 1** the ''General Introduction'', epilepsy in general and absence epilepsy in particular were introduced, and the animal model used in this thesis, the WAG/Rij rat model, was placed in its context. Next, glutamate receptors were introduced, while

literature regarding the relation of epilepsy and glutamate receptors of the metabotropic type were reviewed, narrowing this topic to group I mGluR. Also, an overview of possible clinical applications were given. Lastly, the involvement of group I mGluR in the pathophysiology of absence epilepsy was described.

In **Chapter 2**, the role of mGluR1 in the occurrence of SWDs, the expression, and signaling of mGlu1 receptors were evaluated in thalamic nuclei. This study showed that the mGlu1 PAM, RO0711401 decreased the number of SWD in WAG/Rij rats and slightly increased spontaneous motor activity (not significantly). To further prove a protective role for mGlu1 receptors against SWDs, the same animal model was treated with the mGlu1 NAM, JNJ16259685. The outcome showed an increase in the number of SWDs without any effect on spontaneous motor activity. Next the expression and signaling of mGlu1 receptors was evaluated and the results showed a reduction in the mGluR1 function and expression in the thalamus of symptomatic WAG/Rij rats when compared to non-epileptic control rats. Therefore, it is possible that the effects of the PAM, in terms of SWD occurrence, is due to a hypo-function of mGlu1 receptors in the thalamus. In any case, it is proposed that the PAM of this group could be further developed as an anti-absence agent.

Next, in **Chapter 3**, the mGlu5 receptors function, the expression, the pharmacological manipulation with a PAM, VU0360172, and of the rat's behavior were evaluated. A decrease in both the mGlu5 receptor protein levels and mGlu5-receptor function in the thalamus was found when compared to the age matched non-epileptic ACI rats. Conversely, the expression of mGlu5 receptors was enhanced in the motor cortex and in the somatosensory cortex without an accompanying change in mGlu5-receptor function when compared to ACI rats. It is proposed that VU0360172 might reduce SWDs. It was found that VU0360172 significantly reduced SWDs, without affecting motor behavior. To answer whether this mGlu5 PAM was selective versus mGlu5 receptors, VU0360172 was administered in WAG/Rij rats, pretreated with the antagonist MTEP. It was found that the effect of VU0360172 was prevented by co-treatment with MTEP, demonstrating that the drug decreased absence seizures by amplifying the endogenous activation of mGlu5 receptors. Whether or not the pharmacological effects are due to a hypo-functional thalamus needs to be determined. It now seems that both group I PAMs (RO0711401 and VU0360172) could be further developed as anti-absence drugs.

In the experiments as described in **Chapter 4**, it has been evaluated whether RO0711401 and VU0360172 develop tolerance. This is important since treatment with antiepileptic drugs will always be in chronic cases. Rats were injected twice daily for 10 consecutive days while the EEG was recorded. The outcome showed that rats developed tolerance to RO0711401 on the third day of treatment and were refractory to the drug two days after treatment withdrawal. In contrast, VU0360172 kept its activity during and after the treatment. Biochemical studies revealed that RO0711401 increased the expression of both mGluR1 and mGluR5 in the thalamus, while in the cortex the drug increased mGlu1

receptor levels only. VU0360172 caused an early and constant increase in mGlu5 receptor expression in the thalamus, a late decrease of mGlu5 receptor expression in the cortex, and no changes in mGlu1 receptors. It is proposed that there is a prevalence of mGlu1-mGlu1 homodimers and mGlu1-mGlu5 heterodimers over mGlu5-mGlu5 homodimers in the cortex and thalamus of WAG/Rij. This could explain why the mGlu1 receptor PAM, RO0711401, caused adaptive changes in both mGluR1 and mGluR5, whereas the mGlu5 receptor PAM, VU0360172, selectively induced changes in mGlu5 receptors only.

In **Chapter 5**, a C-T-C circuit demarcation strategy has been adopted by bilateral intra-cortex or intra-thalamus micro-infusions of RO0711401 and VU0360172 respectively to elucidate site-specific effects on the regulation of SWDs. The study showed that the two drugs were equally effective in decreasing SWDs when administered into the cortex; in contrast, the mGlu5 PAM, VU0360172, displayed a greater efficacy than the mGlu1 PAM, RO0711401, when administered into the thalamus. Next to the role of glutamate, GABA also play an important role in the control of SWDs. It is known that systemically administered GABA-mimetic, such as tiagabine, acting by blocking the reuptake of GABA via the high affinity GABA transporter, GAT-1, aggravates the incidence of SWDs. Therefore, by the use of tiagabine, we explored whether, and in which direction, an enhanced availability of extra-synaptic GABA might influence responses to VU0360172, in the thalamus and somatosensory cortex as well. Firstly, tiagabine infused in the cortex dose dependently decreased SWDs. It is thought that by blocking the reuptake of GABA, it is possible to enhance GABA-ergic inhibition. Whereas, tiagabine infused in the thalamus enhanced SWDs. It is proposed that thalamic administered tiagabine facilitated SWD by increasing tonic-inhibition of thalamic relay neurons by inhibiting GABA re-uptake via the high affinity GABA transporter GAT-1.The combination of VU0360172 plus tiagabine in the cortex displayed a SWDs reduction. Instead, the combination of VU0360172 plus tiagabine in the thalamus produced fascinating results. After co-administration, initially the SWD reducing effects of VU0360172 prevailed since the incidence of SWDs in the early post-injection time (10 min) was decreased. Next, this effect was lost at 20 min, and a pro-absence effect at 30 and 40 min occurred. Therefore, we proposed that the reduced SWDs seen in the cortex occurs by enhancing GABA-ergic inhibition onto pyramidal neurons. The intriguing results after the drugs were combined in the thalamus suggest that the activity of mGlu5 receptors is modulated by GABAergic transmission.

In **Chapter 6**, "Is there a future for mGlu5 PAMs in absence epilepsy?" A comparison between ETX, which has become the drug of choice in the treatment of patients with absence seizures taking into account both its tolerability, efficacy, and anti-epileptogenic properties in human and in genetic absence models, and VU0360172 has been made. Here, the need for new treatment options in absence epilepsy was emphasized since 47% of subjects treated with ETX failed in therapy. The assumed working mechanisms of ETX, the neurochemical and pharmacological studies of the new anti-absence drugs mGlu1 and mGlu5 PAMs, RO0711401 and VU0360172, were described and also a comparison

between ETX and VU0360172 was made. The main conclusion of this chapter was that, although both ETX and VU0360172 decreased SWDs when administered in the cortex and thalamus, VU0360172 targets both locations equally effectively, and this is less the case for ETX. This outcome gives us reason to believe that VU0360172 could be successful in seizure suppression in patients.

In **Chapter 7**, the anti-absence and anti-epileptogenetic effects of ETX, and the interaction between VU0360172 and ETX, has been evaluated. Moreover, considering that epileptogenesis and chronic ETX treatment, inducing antiepileptogenesis, might affect mood and cognitive processes such as learning and memory. These latter processes were investigated after chronic ETX treatment as well. The study showed that the anti-absence and anti-epileptogenic effects were successfully induced by ETX. VU0360172, as expected, reduced the SWDs in the untreated control group. However, VU0360172 in chronically ETX treated rats kept the SWD incidence low throughout the whole EEG recording session without returning back to the baseline. This effect occurred both during and after chronic treatment were ended. This data may suggest that a kind of synergism between ETX and VU0360172 has occurred.

Many AEDs, such as ETX, valproic acid, and lamotrigine, have a negative impact on cognition. Therefore it has also been investigated in Chapter 7 if a cognitive aspect, in this case cued spatial discrimation learning in a Y-maze, has been modified after chronic ETX treatment. The outcome showed that chronic ETX enhanced motivation to collect sucrose pallets in the Y-maze, and this was followed by an increase in cued discrimination learning.

In **Chapter 8**, the "General Discussion", the most important findings of the experimental chapters were summarized and their implications were given. First, pharmacological manipulation of group I mGluR was shown to differentially affect SWD activity. Systemic administration of mGlu agonist drugs (RO0711401 and VU0360172) resulted in a decrease of SWD activity, while the antagonist in an increased SWD activity. In the chronic treatment study, tolerance developed against mGlu1 PAM, RO0711401, while tolerance was not developed for the mGlu5 PAM, VU0360172. It was also shown that the function of mGlu1 and mGlu5 receptors in two genetic absence models (Ih/Ih mice and WAG/Rij rats) was not similar, and the role of mGlu receptor PAMs/NAMs is dependent on the genetic background of the animals. It has also shown where within the C-T-C circuit activation of either mGlu1 or mGlu5 receptors (cortex or thalamus) SWDs were reduced. The two drugs were equally effective in reducing SWDs when injected into the cortex; in contrast, the mGlu5 PAM, VU0360172, displayed a greater efficacy than the mGlu1 PAM, RO0711401, when injected into the thalamus. In this thesis it has also been shown which brain area—cortex or thalamus—is involved in the anti-absence and pro-absence effect of tiagabine. Tiagabine micro-infused in the cortex reduced SWDs, while in the thalamus it increased SWDs. The combination of VU0360172 and tiagabine micro-infused in the thalamus but not in the cortex showed that mGlu5 receptors might play a role also through GABAergic

transmission, with mGluR5 also able to control the activity of the GABA transporter (GAT-1). The combination study of ETX plus VU0360172 showed a better pharmacological profile in terms of SWD diminishment when compared to ETX alone. It is also shown that VU0360172 could be combined with ETX since the combination of the two drugs kept the SWD incidence low throughout the whole recording session without returning to the baseline. Future studies are suggested regarding the development of VU0360172 as an anti-absence drug. If the group I PAM receptor can be further developed as a new anti-absence drug, the preclinical profile of VU0360172 in other generalized and focal epilepsy models, including mesial temporal epilepsy, needs to be determined. The fact that it may aggravate the action of GABA-ergic interneurons, as was settled in the focal excitable area in the WAG/Rij model, could point toward this drug being effective in treating other types of epilepsy as well.

# Nederlandse Samenvatting

Absence-epilepsie op kinderleeftijd (childhood absence epilepsy, verder CAE genoemd) betreft 2% tot 8% van de patiënten met epilepsie. De belangrijkste oorzaak is hoofdzakelijk genetisch. In ongeveer een derde van de gezinnen met kinderen met CAE is in de familiegeschiedenis sprake van vergelijkbare gevallen. De broers en zussen van kinderen met CAE lopen een kans van ongeveer 10% om ook epilepsie te krijgen. CAE wordt gezien als een van de relatief goedaardige vormen van kinderepilepsie. Deze aandoening begint meestal op een leeftijd van vier tot acht jaar en wordt gekenmerkt door dagelijkse, regelmatig terugkerende, korte periodes van staren, waarbij het kind vaak knipperende oogleden heeft en niet reageert op externe stimuli. Er zijn dringend nieuwe geneesmiddelen tegen epilepsie nodig, ook gezien het feit dat niet alle proefpersonen (57% à 74%) vrij is van aanvallen en dat een behandeling met ethosuximide (ETX), een van de voorkeursgeneesmiddelen tegen absences, faalt bij ongeveer 47% van de proefpersonen.

Nieuwe geneesmiddelen tegen absences kunnen zowel ingrijpen in de glutamaterge en/of GABA-erge neurotransmissie, de beide hoofdrolspelers in het netwerk dat betrokken is bij de cortico-thalamo-corticale oscillaties (C-T-C-oscillaties) die verantwoordelijk zijn voor de zeer stereotype piekgolfontladingen (spike-wave discharges, verder te noemen SWD's). Op grond van knaagdiermodellen is inmiddels aangetoond dat het faciale gedeelte van de somatosensorische cortex beschikt over een hyperexciteerbaar gebied waar de SWD's hun oorsprong hebben. De aanwezigheid van metabotrope glutamaatreceptoren (mGluRs) binnen het C-T-C-circuit is een aanwijzing dat deze receptoren een mogelijk target zijn bij de behandeling van absence-epilepsie. Bovendien is er bij mGluRs als target van behandeling minder sprake van toxiciteit dan bij ionotrope glutamaatreceptoren het geval zou zijn. Het was daarom absoluut geboden om te verkennen welke rol mGluRs speelden bij absence-epilepsie, ook omdat geneesmiddelen die mGluR als target hadden in verschillende modellen voor toevallen en epilepsie een pro- of anti-epileptische werking vertoonden. Een laatste reden was daarnaast nog dat allostere modulatoren uit groep I veilig zijn gebleken en zich ook al in de klinische ontwikkelingsfase bevinden voor behandelingen van andere neurologische en psychiatrische aandoeningen.

Er zal in het bijzonder gekeken worden naar de rol van mGlu-receptoren die behoren tot groep I — zowel mGlu1 als mGlu5 behoren tot deze categorie. Ze komen preferentieel tot expressie in het perifere gedeelte van de postsynaptische verdichting, waar ze excitatieresponsen oproepen en controle uitoefenen op mechanismen van synaptische plasticiteit.

Bij de experimenten die in dit proefschrift beschreven worden, is onderzoek gedaan naar de betrokkenheid van mGluRs van groep I in de pathofysiologie van absence-epilepsie. Dit werd gedaan in een goed gevalideerd en veelgebruikt model: ratten van de WAG/Rij-stam. De rol van mGluR1 en mGluR5 is onderzocht door vaststelling van hun expressie en signalering in de hersengebieden waar zich de pathologische SWD's voordoen, waar ze zich vanuit verspreiden en waarin ze in stand worden gehouden: het C-T-C-circuit. In de

meeste gevallen werden ratten met symptomen vergeleken met ratten zonder symptomen en met ACI-ratten in dezelfde leeftijdsgroep. Bovendien werd er farmacologisch EEG-gedragsonderzoek uitgevoerd, met als doel om vast te stellen of positieve allostere modulatoren (PAM's) of negatieve allostere modulatoren (NAM's) uit deze groep mogelijk kunnen worden ingezet als geneesmiddel tegen absences.

In **Hoofdstuk 1**, de 'Algemene inleiding', wordt een introductie gegeven tot epilepsie in het algemeen en absence-epilepsie in het bijzonder. Daarnaast wordt het diermodel dat in dit promotieonderzoek is gebruikt, het WAG/Rij-rattenmodel, in de juiste context geplaatst. Daarna worden de glutamaatreceptoren geïntroduceerd en wordt ingegaan op literatuur over het verband tussen epilepsie en glutamaatreceptoren van het metabotrope type. Daarna spitst dit onderwerp zich toe op mGluRs in groep I. Vervogens wordt een overzicht gegeven van mogelijke klinische toepassingen. Ten slotte volgt er een omschrijving van de wijze waarop mGluRs uit groep I betrokken zijn bij de pathofysiologie van absence-epilepsie.

In **Hoofdstuk 2** wordt de rol besproken die mGluR1 speelt bij het optreden van SWD's en wordt ingegaan op de expressie en signalering van mGlu1-receptoren in de thalamuskernen. In dit onderzoek is aangetoond dat de mGlu1-PAM RO0711401 het aantal SWD's in WAG/Rij-ratten verminderde en de spontane motorische activiteit iets deed toenemen (niet-significant). Om verder aan te tonen dat mGlu1-receptoren bescherming kunnen bieden tegen SWD's, werd hetzelfde diermodel ook behandeld met mGlu1-NAM JNJ16259685. Het resultaat hiervan was een toename van het aantal SWD's, zonder dat er sprake was van een effect op de spontane motorische activiteit. Vervolgens wordt de expressie en signalering van mGlu1-receptoren besproken en wordt gewezen op de resultaten, waaruit blijkt dat er bij WAG/Rij-ratten met symptomen sprake is van een vermindering in de werking en expressie van mGluR1 in de thalamus vergeleken met de niet-epileptische ratten uit de controlegroep. Het is dan ook mogelijk dat de effecten van de PAM, gemeten naar het optreden van SWD's, het gevolg zijn van een hypofunctie van de mGlu1-receptoren in de thalamus. In elk geval wordt het voorstel gedaan om te onderzoeken of de PAM's uit deze groep verder kunnen worden ontwikkeld tot een middel tegen absences.

In **Hoofdstuk 3** wordt ingegaan op de werking van de mGlu5-receptor, op de expressie en de farmacologische manipulatie ervan met behulp van een PAM, VU0360172, en op het gedrag van de rat. Vergeleken met de niet-epileptische ACI-ratten uit dezelfde leeftijdsgroep was er sprake van een afname in zowel de receptoreiwitspiegel van mGlu5 als van de werking van de mGlu5-receptor in de thalamus. Tegengesteld daaraan was er in vergelijking met ACI-ratten sprake van sterkere expressie van de mGlu5-receptoren in de motorische  en in de somatosensorische cortex, zonder dat dit gepaard ging met een verandering in de werking van de mGlu5-receptor. Er wordt het vermoeden geuit dat VU0360172 SWD's zou kunnen verminderen. Er werd vastgesteld dat VU0360172 zorgde voor een significante vermindering van de SWD's, zonder dat dit van invloed was op het

motorische gedrag. Om te kunnen beantwoorden of deze mGlu5-PAM selectief was voor mGlu5-receptoren, kregen met de antagonist MTEP voorbehandelde WAG/Rij-ratten VU0360172 toegediend. Er werd vastgesteld dat bij concomitante behandeling met MTEP de werking van VU0360172 werd tegengegaan. Hiermee werd aangetoond dat het geneesmiddel absence-toevallen verminderde door versterking van de endogene activering van de mGlu5-receptoren. Of de farmacologische effecten het gevolg zijn van een hypofunctie van de thalamus of niet moet nog worden bepaald. Naar nu blijkt kunnen PAM's uit groep I (RO0711401 en VU0360172) mogelijk verder worden ontwikkeld tot geneesmiddelen tegen absences.

Tijdens de experimenten die in **Hoofdstuk 4** beschreven worden, is nagegaan of er bij RO0711401 en VU0360172 sprake is van de ontwikkeling van tolerantie. Dit is een punt dat van groot belang is, aangezien behandelingen met geneesmiddelen tegen epilepsie altijd in chronische gevallen zullen worden toegepast. Ratten werden twee keer per dag gedurende tien achtereenvolgende dagen geïnjecteerd en er werden EEG's gemaakt. Het resultaat was dat de ratten op de derde dag van de behandeling een tolerantie ontwikkelden voor RO0711401 en twee dagen na intrekken van de behandeling farmacoresistent waren. De werking van VU0360172 bleef echter wel in stand, zowel tijdens als na de behandeling. Uit biochemisch onderzoek bleek dat bij RO0711401 zowel mGluR1 als mGluR5 sterker tot expressie kwam in de thalamus, terwijl het geneesmiddel in de cortex alleen de spiegel van de mGlu1-receptor deed stijgen. VU0360172 veroorzaakte een vroege en constante toename in de expressie van de mGlu5-receptor in de thalamus, een late afname van de expressie van de mGlu5-receptor in de cortex en geen veranderingen in de mGlu1-receptoren. Er wordt aangenomen dat er in de cortex en de thalamus van WAG/Rij-ratten sprake is van een prevalentie van mGlu1-mGlu1-homodimeren en mGlu1-mGlu5-heterodimeren ten opzichte van mGlu5-mGlu5-homodimeren. Dit zou een verklaring kunnen zijn waarom de mGlu1-receptor-PAM RO0711401 zowel in mGluR1 als in mGluR5 adaptieve veranderingen tot stand bracht, terwijl de mGlu5-receptor-PAM VU0360172 alleen selectieve veranderingen teweegbracht in de mGlu5-receptoren.

In **Hoofdstuk 5** wordt beschreven hoe er een demarcatiemethode in het C-T-C-circuit tot stand werd gebracht door middel van bilaterale intra-corticale of intra-thalamische micro-infusies met respectievelijk RO0711401 en VU0360172, om zo de specifieke plaatselijke effecten op de regulering van SWD's duidelijker te kunnen bepalen. Uit het onderzoek bleek dat beide geneesmiddelen bij toediening in de cortex in gelijke mate effectief waren bij de vermindering van SWD's. Bij toediening in de thalamus bleek de mGlu5-PAM VU0360172 effectiever te zijn dan de mGlu1-PAM RO0711401. Naast de rol van glutamaat speelt ook GABA een belangrijke rol bij de beperking van SWD's. Het is bekend dat een systemisch toegediend GABA-mimeticum tot een toename in de incidentie van SWD's leidt. Een voorbeeld van een dergelijk GABA-mimeticum is tiagabine, waarvan de werking zorgt voor blokkering van de heropname van GABA via GAT-1, een GABA-transporteiwit met een hoge affiniteit. Door toepassing van tiagabine verkenden wij in

hoeverre en in welke richting een grotere beschikbaarheid van extra-synaptisch GABA de respons op VU0360172 beïnvloedt, zowel in de thalamus als in de somatosensorische cortex. Bij infusie in de cortex verminderde tiagabine het aantal SWD's dosis afhankelijk. Er bestaat het vermoeden dat het door blokkering van de heropname van GABA mogelijk is om de GABA-erge inhibitie te versterken. De infusie van tiagabine in de thalamus deed het aantal SWD's echter toenemen. Er wordt aangenomen dat de toediening van tiagabine in de thalamus bevorderlijk was voor SWD's door een toename van de tonische inhibitie van schakelneuronen in de thalamus, als gevolg van inhibitie van de heropname van GABA via GAT-1. De combinatie van VU0360172 plus tiagabine in de cortex leidde tot een vermindering van de SWD's. De combinatie van VU0360172 plus tiagabine leidde in de thalamus echter tot fascinerende resultaten. Na concomitante toediening overheerste in eerste instantie het dempende effect op SWD's van VU0360172, aangezien de incidentie van SWD's afnam in de vroege periode post-injectie (10 min.). Dit effect ging echter na 20 min. verloren en na 30 en 40 min. was er sprake van een pro-absence-effect. Wij veronderstelden dan ook dat de afname van SWD's in de cortex het gevolg is van een versterking van de GABA-erge inhibitie op de piramidale neuronen. De intrigerende resultaten na combinatie van de geneesmiddelen in de thalamus doen vermoeden dat de activiteit van mGlu5-receptoren wordt gemoduleerd door GABA-erge transmissie.

In **Hoofdstuk 6**, "Is er een toekomst voor mGlu5-PAM's bij absence-epilepsie?", wordt er een vergelijking gemaakt tussen VU0360172 en ETX, een geneesmiddel dat is uitgegroeid tot de voorkeursbehandeling bij patiënten met absence-toevallen, rekening houdende met zowel de verdraagbaarheid, werking en anti-epileptogene eigenschappen in mensen en in genetische absence-modellen. Hierbij werd duidelijk hoezeer er nieuwe behandelmogelijkheden nodig zijn bij absence-epilepsie, aangezien bij 47% van de proefpersonen die met ETX behandeld werden, de behandeling niet aansloeg. Er wordt een beschrijving gegeven van de vermoedelijke werkingsmechanismen van ETX, de neurochemische en farmacologische onderzoeken naar de nieuwe anti-absence-geneesmiddelen RO0711401 en VU0360172, de mGlu1- en mGlu5-PAM's, en daarnaast wordt er een vergelijking gemaakt tussen ETX en VU0360172. De belangrijkste conclusie van dit hoofdstuk is dat weliswaar zowel ETX als VU0360172 bij toediening in de cortex en de thalamus zorgden voor een afname van SWD's, maar dat VU0360172 op beide locaties even effectief bleek te zijn, wat bij ETX slechts in mindere mate het geval is. Dit resultaat is voor ons een reden om aan te nemen dat VU0360172 succesvol bij patiënten zou kunnen worden ingezet voor het onderdrukken van toevallen.

In **Hoofdstuk 7** wordt gekeken naar de anti-absence-effecten en anti-epileptogene effecten van ETX en naar de interactie tussen VU0360172 en ETX. Bovendien werd gekeken naar de effecten van epileptogenese en chronische behandelingen met ETX, waardoor anti-epileptogenese tot stand komt, op iemands stemming en op cognitieve processen, zoals leer- en geheugenprestaties. Naar deze laatste processen werd ook onderzoek gedaan naar een chronische behandeling met ETX. Uit het onderzoek bleek

dat de anti-absence-effecten en anti-epileptogene effecten door middel van ETX met succes kunnen worden opgeroepen. Zoals verwacht verminderde VU0360172 de SWD's in de onbehandelde controlegroep. Bij ratten die chronisch waren behandeld met ETX, bleek VU0360172 de incidentie van SWD's echter laag te houden gedurende de hele periode van EEG-registratie, zonder terug te keren naar de beginwaarde. Dit effect hield zowel tijdens als na afloop van de chronische behandeling aan. Uit deze gegevens blijkt dat er mogelijk sprake is van een soort synergie tussen ETX en VU0360172.

Veel anti-epileptische geneesmiddelen, zoals ETX, valproïnezuur en lamotrigine, hebben een negatief effect op de cognitie. Daarom is in hoofdstuk 7 ook onderzoek gedaan naar de vraag of een cognitief aspect, in dit geval een impulsgestuurde leertaak op het gebied van ruimtelijk onderscheidingsvermogen in een Y-vormig doolhof ('Y-maze'), na een chronische behandeling met ETX veranderingen heeft ondergaan. Het resultaat was dat chronische ETX de motivatie versterkte om sucrosekorrels op te halen in het Y-vormige doolhof, gevolgd door een toename in impulsgestuurd leren ten aanzien van het onderscheidingsvermogen.

In **Hoofdstuk 8**, de 'Algemene discussie', worden de belangrijkste bevindingen uit de hoofdstukken met beschrijvingen van de experimenten samengevat en worden de implicaties genoemd. Ten eerste bleek de SWD-activiteit door farmacologische manipulatie van mGluRs uit groep I op verschillende manieren te kunnen worden beïnvloed. Systemische toediening van de mGlu-agonisten (RO0711401 en VU0360172) leidde tot een afname van de SWD-activiteit, terwijl de antagonist de SWD-activiteit deed toenemen. In het onderzoek naar chronische behandelingen bleek zich tolerantie te ontwikkelen voor de mGlu1-PAM RO0711401, terwijl dit niet het geval was mGlu5-PAM VU0360172. Er werd ook aangetoond dat de werking van mGlu1- en mGlu5-receptoren in twee genetische absence-modellen (lh/lh-muizen en WAG/Rij-ratten) niet vergelijkbaar was en dat de rol van de mGlu-receptor-PAM's/NAM's afhangt van de genetische achtergrond van de dieren. Er is ook aangetoond waar in het C-T-C-circuit de activering van de mGlu1- of mGlu5-receptoren (cortex of thalamus) leidde tot een afname van de SWD's. Beide geneesmiddelen waren bij injectie in de cortex in gelijke mate effectief bij de vermindering van SWD's. Bij injectie in de thalamus bleek de mGlu5-PAM VU0360172 effectiever te zijn dan de mGlu1-PAM RO0711401. In dit promotieonderzoek is ook aangetoond welk hersengebied – cortex of thalamus – betrokken is bij de anti-absence- en pro-absence-effecten van tiagabine. Micro-infusie van tiagabine in de cortex verminderde het aantal SWD's, terwijl er bij micro-infusie in de thalamus sprake was van een toename van de SWD's. De combinatie van VU0360172 en micro-infusie van tiagabine in de thalamus toonde aan dat mGlu5-receptoren mogelijk ook een rol spelen in de vorm van GABA-erge transmissie, waarbij mGluR5 ook in staat is om de activiteit van het GABA-transporteiwit (GAT-1) te controleren. Dit effect deed zich niet voor in de cortex. Uit het onderzoek naar de combinatie van ETX plus VU0360172 kwam naar voren dat het farmacologische profiel, in termen van vermindering van SWD's, beter was in vergelijking

met alleen ETX. Er werd ook aangetoond dat VU0360172 zou kunnen worden gecombineerd met ETX, aangezien de combinatie van de beide geneesmiddelen de incidentie van SWD's gedurende de hele registratiesessie laag hield, zonder terug te keren naar de beginwaarde.

Aanbevolen wordt om verder onderzoek te doen naar de ontwikkeling van VU0360172 tot een geneesmiddel tegen absences. Als de PAM-receptor van groep I verder kan worden ontwikkeld tot een nieuw anti-absencegeneesmiddel, dan moet het preklinische profiel van VU0360172 ook worden bepaald voor andere gegeneraliseerde en focale epilepsiemodellen, waaronder mesiale temporaalkwabepilepsie. Het feit dat dit geneesmiddel in staat bleek om de werking van GABA-erge interneuronen te versterken, zoals bleek uit het focale exciteerbare gebied in het WAG/Rij-model, kan erop wijzen dat dit geneesmiddel ook effect kan hebben bij de behandeling van andere vormen van epilepsie.

# Curriculum Vitae

Valerio D'Amore was born on 23rd of June 1986 in Catania. After finishing his secondary school (lyceum) Leonardo Da Vinci also in Catania in 2004, he started in the same year his study in pharmacy at the University of Catania. He obtained his Master degree in 2009 with his thesis entitled "Switch in the expression of mGlu1 and mGlu5 metabotropic glutamate receptors in the cerebellum of mice developing experimental autoimmune encephalomyelitis and in autoptic cerebellar samples from patients with multiple sclerosis". He started in 2010 his residency at the School of Clinical Pharmacology at the University of Roma, la Sapienza, He obtained his residency in 2015. One year after he started his residency, he started also his doctoral studies in 2011 at Neuromed, under the supervision of Prof. dr. Ferdinando Nicoletti and dr. Richard Teke Ngomba. One year later he became a fellow PhD student at the Donders Center for Cognition, Radboud University Nijmegen. Under the supervison of Prof. dr. Gilles van Luijtelaar, and dr. Tineke van Rijn he worked on a PhD project investigating the role of group I metabotropic receptors in absence epilepsy.

# Attended Conferences

2015 Neuronus IBRO-IRUN Neuroscience forum, Krakow, Poland
2014 Dutch Endo-Neuro–Psycho meeting, Lunteren, Netherlands
2014 Neuronus IBRO-IRUN Neuroscience forum, Krakow, Poland
2014 Lega Italiana contro l' Epilessia (LICE)Triest, Italy
2014 Neuronus IBRO & IRUN Neuroscience Forum, Krakow, Poland
2014 Society for Neuroscience, Washington DC, USA
2014 International meeting on Metabotropic Glutamate receptors, Taormina, Italy
2013 Society for Neuroscience, San Diego, USA
2012 Society for Neuroscience, New Orleans, USA
2012 Neurons IBRO & IRUN Neuroscience Forum, Krakow, Poland
2011 International meeting on Metabotropic Glutamate receptors, Taormina, Italy
2010 Society for Neuroscience, San Diego, USA

# List of publications

## International peer reviewed journals

**D'Amore V,** von Randow C, Nicoletti F, Ngomba RT, van Luijtelaar G. 2015. Anti-absence activity of mGlu1 and mGlu5 receptor enhancers and their interaction with a GABA reuptake inhibitor: Effect of local infusions in the somatosensory cortex and thalamus. Epilepsia. 56(7):1141-51.

**D'Amore V,** Renée H L Raaijmakers, Ines Santolini, Clementina M van Rijn, Richard Teke Ngomba, Ferdinando Nicoletti, Gilles van Luijtelaar. 2015. The antiabsence effect of mGlu5 receptor amplification with VU0360172 is maintained in WAG/Rij rats chronically treated with ethosuximide. Pharmacology, Biochemestry and Behavior. Accepted. Pending minor revision.

**D'Amore V,** Santolini I, Celli R, Lionetto L, De Fusco A, Simmaco M, van Rijn CM, Vieira E, Stauffer SR, Conn PJ, Bosco P, Nicoletti F, van Luijtelaar G, Ngomba RT. 2014. Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats. Neuropharmacology. 85:91-103.

**D'Amore V,** Santolini I, van Rijn CM, Biagioni F, Molinaro G, Prete A, Conn PJ, Lindsley CW, Zhou Y, Vinson PN, Rodriguez AL, Jones CK, Stauffer SR, Nicoletti F, van Luijtelaar G, Ngomba RT. 2013. Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacology. 66:330-8.

Ngomba RT, Santolini I, Biagioni F, Molinaro G, Simonyi A, van Rijn CM, **D'Amore V,** Mastroiacovo F, Olivieri G, Gradini R, Ferraguti F, Battaglia G, Bruno V, Puliti A, van Luijtelaar G, Nicoletti F. 2011. Protective role for type-1 metabotropic glutamate receptors against spike and wave discharges in the WAG/Rij rat model of absence epilepsy. Neuropharmacology. 60(7-8):1281-91.

## Book Chapter

Gilles van Luijtelaar, **Valerio D'Amore,** Ines Santolini, Richard Ngomba. Is there a future for mGlu5 PAMs in absence epilepsy? A comparison with Ethosuximide. Submitted to "The Receptors": mGlu Receptors, 2015, eds: Nicoletti, Battaglia, Ngomba, and Di Giovanni. Springer Publisher (submitted).

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